COMPOSITIONS AND METHODS FOR DETECTING SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS

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FIELD OF THE INVENTION

The invention relates to compositions and methods for detecting the presence of severe acute respiratory syndrome (SARS)-coronavirus, and for screening anti-SARS coronavirus agents and vaccines. The invention also relates to reducing infection with plus-strand RNA viruses such as SARS-coronavirus. These methods may be used for increasing the safety of cell cultures that are used in screening clinical samples for respiratory pathogens other than SARS-coronavirus.

BACKGROUND OF THE INVENTION

An outbreak of severe acute respiratory syndrome (SARS) emerged in Guangdong Province, People's Republic of China in November 2002. From China, SARS spread to 30 other countries and as of August 7, 2003, this outbreak resulted in 8,422 reported cases, of which 918 were fatal. Through the coordinated efforts of laboratories around the world, a novel coronavirus, SARS-coronavirus (SARS-CoV), was identified as the causative agent of SARS (Drosten, et al., 2003, N.Engl.J.Med. 348:1967-1976; Fouchier, et al., 2003, Nature 423:240; Ksiazek, et al., 2003, N.Engl.J.Med. 348:1953-1966; Peiris, et al., 2003, Lancet 361:1319-1325; Poutanen, et al., 2003, N.Engl.J.Med. 348:1995-2005). This discovery was quickly followed by the publication of the complete genomic sequences of two SARS-CoV isolates and identification of specific subgenomic RNAs and proteins involved in replication (Marra, et al., 2003, Science 300:1399-1404; Rota, et al., 2003, Science 300:1394-1399; Thiel, et al., 2003, J.Gen.Virol. 84:2305-2315). Phylogenetic analysis of the SARS-CoV *replicase* gene demonstrated that despite a number of unique features, SARS-CoV is most closely related to group 2 coronaviruses, which include mouse hepatitis virus (MHV), bovine coronavirus (BCoV) and human coronavirus OC43 (HCoV-OC43) (Snijder, et al., 2003, J.Mol.Biol. 331:991-1004).

SARS-CoV has been detected using the Vero E6 cell line and fetal rhesus monkey kidney cells (the only cell lines reported to be susceptible to SARS-CoV). Susceptibility of these cells to SARS-CoV was based on observing a cytopathic effect (CPE) post inoculation with SARS-CoV. However, many coronaviruses cause persistent infections in cell cultures and some show little evidence of CPE. Thus, using CPE to identify entry of SARS-CoV or abortive replication is insensitive, misleading, and does not correctly identify virus entry and/or replication.

SARS-CoV has also been detected using virus titration techniques, RT-PCR specific to SARS-CoV genomic RNA, and immunofluorescence assay. However, these methods are laborious, and do not distinguish between entry and replication of the virus.

Thus, there remains a need for compositions and methods for detecting the presence of SARS-coronavirus, for screening anti-SARS coronavirus agents and vaccines. There is also a need for increasing the safety of cell cultures that are routinely used in laboratories and that may support infection by plus-strand RNA viruses, such as SARS-coronavirus.

SUMMARY OF THE INVENTION

The invention provides compositions and methods for detecting the presence of SARS-coronavirus, and for screening anti-SARS coronavirus agents and vaccines. Also provided are compositions and methods for reducing infection with plus-strand RNA viruses such as SARS-coronavirus.

In one embodiment, the invention provides a method for detecting replication of severe acute respiratory syndrome coronavirus (SARS-coronavirus) in a sample, comprising detecting the presence SARS-coronavirus sgRNA in a sample. In one example, sgRNA comprises at least a portion of a leader sequence. In another example, the sgRNA comprises a gene encoding a SARS-coronavirus polypeptide. In another embodiment, the method further comprises detecting SARS-coronavirus gRNA. While not intending to limit the method of detection, in one embodiment, the detecting of gRNA and/or sgRNA is by reverse transcriptase PCR, ribonuclease protection assay, and/or by Northern blot. In another embodiment, the method further comprises quantitating sgRNA and/or gRNA. In yet a further embodiment, the method further comprises detecting one or more SARS-coronavirus polypeptide using, for example, immunofluorescence and/or Western blot. In an additional embodiment, the method further comprises detecting SARS-coronavirus particles.

. The invention also provides a method for detecting the presence of severe acute respiratory syndrome coronavirus (SARS-coronavirus) in a sample, comprising: a) providing: (i) a sample; and (ii) cells, wherein said cells support replication of SARS-coronavirus in the absence of substantial cytopathic effect; b) inoculating the cells with the sample to produce inoculated cells; and c) detecting the presence of the SARS-coronavirus in the inoculated cells. In some preferred embodiments, the cells are chosen from but not limited to HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK. In one embodiment, the detecting step comprises detecting the presence of a SARS-coronavirus polypeptide (such as Nucleocapsid (N), Spike Glycoprotein (S), Matrix (M), E protein, and Replicase proteins) by, for example, immunofluorescence and/or Western blot. Alternatively, or in addition, the detecting may comprise detecting the presence of SARS-coronavirus gRNA and/or sgRNA.

While not intending to limit the type or source of cell in any of the invention's methods, in one embodiment, the cells comprise a transgenic cell and/or wild type cell. In a preferred embodiment, the transgenic cell comprises Mv1Lu-hF deposited as ATCC accession number PTA-4737. Alternatively, the transgenic cell comprises a cell line established from a transgenic cell line designated Mv1Lu-hF, wherein the established cell line has a property selected from the group consisting of (a) increased sensitivity to at least one virus selected from the group consisting of influenza A virus, influenza B virus and parainfluenza virus 3, as compared to the Mv1Lu cell line, and (b) enhanced productivity of infectious virions upon inoculation with at least one virus selected from the group one consisting of influenza A virus, influenza B virus and parainfluenza virus 3, as compared to the Mv1Lu cell line. In another embodiment, the transgenic cell comprises a transgenic mink lung epithelial cell line expressing human furin, wherein the cell line has a property selected from the group consisting of (a) increased sensitivity to at least one virus selected from the group consisting of influenza A virus, influenza B virus and parainfluenza virus 3, as compared to Mv1Lu, and (b) enhanced productivity of infectious virions upon inoculation with at least one virus selected from the group one consisting of influenza A virus, influenza B virus and parainfluenza virus 3, as compared to Mv1Lu. In a further embodiment, the inoculating step comprises contacting the cells (whether wild type and/or transgenic) with a protease inhibitor.

While not intending to limit the type of culture in any of the invention's methods, the cells may be in single cell type culture, in mixed cell type culture with a second cell type,

and/or are frozen *in situ*. Also without limiting the source or type of sample in any of the invention's methods, sample is isolated from a mammal, preferably from a human.

The invention further provides a method for detecting the presence of severe acute respiratory syndrome coronavirus (SARS-coronavirus) in a first sample and in a second sample, comprising: a) providing: (i) a first sample; (ii) a second sample; b) contacting test cells with: (i) the first sample to produce a first treated sample; and (ii) the second sample to produce a second treated sample; wherein the test cells support replication of SARS-coronavirus in the absence of substantial cytopathic effect, and the contacting is under conditions such that the test cells are infected with SARS-coronavirus; c) detecting the presence of SARS-coronavirus gRNA and SARS-coronavirus sgRNA, wherein the detecting indicates the presence of the SARS-coronavirus. In some embodiments, the test cells are chosen from HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK. In one embodiment, the detecting step comprises detecting one or more of: i) absence of SARS-coronavirus gRNA in the first treated sample; ii) reduced level of SARS-coronavirus sgRNA in the first treated sample compared to the level of sgRNA in the second treated sample; and iii) reduced ratio of SARS-coronavirus sgRNA level to SARS-coronavirus gRNA level in the first treated sample compared to in the second treated sample; wherein the detecting indicates that the first sample contains a reduced level of SARS-coronavirus compared to the second sample. In one embodiment, the first sample is from a mammal treated with an agent identified according to any method, and the second sample is from the mammal that is not treated with the agent. In another embodiment, the first sample is from a mammal treated with a first concentration of an agent identified according to any method, and the second sample is from the mammal treated with a second concentration of the agent, wherein the first and second concentrations are different. In yet another embodiment, the first sample is from a mammal treated with a first agent identified according to any method, and the second sample is from the mammal treated with a second agent identified according to any method, wherein the first and second agent are different.

The invention also provides a method for identifying a test agent as altering (such as reducing or increasing) replication of severe acute respiratory syndrome coronavirus (SARS-coronavirus) in a cell, comprising: a) providing cells treated with a first test agent, wherein the cells support replication of SARS-coronavirus in the absence of substantial cytopathic effect; and b) detecting an altered level of replication of cells treated with the first test agent compared to a level of replication of the cells not treated with the first test

agent, wherein the detecting identifies the first test agent as altering replication of severe acute respiratory syndrome coronavirus (SARS-coronavirus) in a cell. In some embodiments, the cells are chosen from HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK. Without limiting the method of detection, in one embodiment, the detecting step may comprise detecting SARS-coronavirus sgRNA, gRNA, polypeptide and/or virus particle. In another embodiment, the detecting comprises detecting one or more of: i) absence of SARS-coronavirus gRNA in the treated cells; ii) reduced level of SARS-coronavirus sgRNA in the treated cells compared to the level of sgRNA in the cells that are not treated with the first test agent; and iii) reduced ratio of SARS-coronavirus sgRNA level relative to SARS-coronavirus gRNA level in the treated cells compared to in the cells that are not treated with the first test agent; wherein the detecting identifies the first test agent as reducing replication of severe acute respiratory syndrome coronavirus (SARS-coronavirus) in a cell. Without limiting the use or methodology, it may be desirable to compare the efficacy of two potential drugs, by comparing their effect on only sgRNA by detecting comprises detecting one or more of: i) reduced level of SARS-coronavirus sgRNA in the cells treated with a second test agent compared to the level of sgRNA in the cells treated with the first test agent; and ii) reduced ratio of SARS-coronavirus sgRNA level to SARS-coronavirus gRNA level in the cells treated with a second test agent compared to the ratio in the cells treated with the first test agent. In an exemplary embodiment, detecting one or more of: a) an increased reduction in the level of SARS-coronavirus sgRNA in the cells treated with the first test agent compared to the cells treated with the second test agent, and b) an increased reduction in the ratio of SARS-coronavirus sgRNA level to SARS-coronavirus gRNA level in the cells treated with the first test agent compared to the cells treated with the second test agent, wherein the detecting identifies the first test agent as more efficacious than the second test agent in reducing replication of severe acute respiratory syndrome coronavirus (SARS-coronavirus) in a cell.

Additionally provided herein is a method for reducing replication of severe acute respiratory syndrome coronavirus (SARS-coronavirus) in a mammal, comprising administering a therapeutic amount of a test agent to the mammal, wherein the test agent is identified according to the above method.

The invention also provides a method for producing one or more of severe acute respiratory syndrome coronavirus (SARS-coronavirus) particles and SARS-coronavirus polypeptide, comprising: a) providing: (i) SARS-coronavirus; and (ii) a cell type chosen

from HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK; and b) inoculating the cell type with the virus under conditions such that the inoculated cell produces one or more of SARS-coronavirus and SARS-coronavirus polypeptide.

Moreover, provided by the invention is an antibody specific for one or more SARS-coronavirus antigen that is produced by a cell chosen from HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK. In some embodiments, the antibody is chosen from a polyclonal antibody, a monoclonal antibody, and a humanized antibody.

Also provided is a severe acute respiratory syndrome coronavirus (SARS-coronavirus) vaccine produced using cells chosen from HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK.

The invention additionally provides a method for immunizing a mammal against severe acute respiratory syndrome coronavirus (SARS-coronavirus), comprising administering to a mammal a vaccine produced according any method, wherein the administering generating an immune response in the mammal against severe acute respiratory syndrome coronavirus (SARS-coronavirus).

Also provided herein is a composition, comprising (i) cells susceptible to a virus that is not a plus-strand RNA virus, and (ii) protease inhibitor. The plus-strand RNA virus is exemplified by Adenovirus, Arenaviridae, Baculoviridae, Birnaviridae, Bunyaviridae, Caliciviridae, Cardiovirus, Corticoviridae, Cystoviridae, Epstein-Barr virus, Enterovirus, Filoviridae, Foot-and-mouth disease virus, Hepadnviridae, Hepatitis virus, Herpesviridae, Immunodeficiency virus, Influenza virus, Inoviridae, Iridoviridae, Orthomyxoviridae, Papovaviru, Paramyxoviridae, Parvoviridae, Poliovirus, Polydnaviridae, Poxyviridae, Reoviridae, Retrovirus, Rhabdoviridae, Rhinoviridae, Semliki Forest virus, Tetraviridae, Toroviridae, Vaccinia virus, and Vesicular stomatitis virus.

Further provided herein is a method for detecting a virus that is not a plus-strand RNA virus in a sample, comprising: a) providing: i) a sample; ii) cells susceptible to the virus that is not a plus-strand RNA virus; and iii) at least one protease inhibitor; b) contacting the cells and the sample in the presence of the protease inhibitor to produce contacted cells, wherein replication of the virus that is not a plus-strand RNA virus in the contacted cells is not reduced relative to replication of the virus that is not a plus-strand RNA virus in cells not contacted with the protease inhibitor, and wherein replication of a plus-strand RNA virus in the cells contacted with the protease inhibitor is reduced relative to replication of the plus-strand RNA virus in cells not contacted with the protease inhibitor.

In one embodiment, the plus-strand RNA virus is chosen from togavirus, flavivirus, coronavirus, and picornavirus.

The invention also provides a composition comprising (i) cells susceptible to a virus chosen from influenza virus, parainfluenza virus, adenovirus, and respiratory syncytial virus, and (ii) protease inhibitor. In some preferred embodiments, the compositions further comprise a cyclodextrin, in a subset of these the cyclodextrin is Captisol. In other preferred embodiments, the protease inhibitor is selected from but not limited to Actinonin, Glycyrrhizin, and E64D.

Also provided herein, is method for detecting a virus chosen from influenza virus, parainfluenza virus, adenovirus, and respiratory syncytial virus in a sample, comprising: a) providing: i) a sample; ii) cells susceptible to the virus; and iii) one or more protease inhibitor; b) contacting the cells and the sample in the presence of the protease inhibitor to produce contacted cells, wherein replication of the contacted cells by the virus is not reduced relative to cells not contacted with the protease inhibitor, and wherein replication of the contacted cells by severe acute respiratory syndrome coronavirus (SARS-coronavirus) is reduced relative to cells not contacted with the protease inhibitor. In one embodiment, the influenza virus is chosen from influenza A, influenza B, and influenza C, the parainfluenza virus is chosen from parainfluenza 1, parainfluenza 2, and parainfluenza 3. Without limiting the cell type in this or any of the invention's methods, in one embodiment, the cells comprise a transgenic cell (such as Mv1Lu-hF) and/or wild type cell. Also without limiting the nature of the culture used in this and in any other of the invention's methods, the cells may be in single cell type culture, mixed cell type culture (comprising a wild type cell and/or a transgenic cell), and/or are frozen *in situ*. In one embodiment, the inoculated Mv1Lu cells are incubated with the sample for up to 24 hours and/or up to 48 hours. Without limiting the type of sample in any of the invention's methods, the sample is isolated from a mammal that has been treated with an agent that is suspected of reducing replication of SARS-coronavirus in a cell.

Moreover, the present invention provides methods for detecting replication of a coronavirus in a sample, comprising detecting the presence of coronavirus subgenomic RNA in a sample by reverse transcriptase polymerase chain reaction (PCR). In preferred embodiments, the subgenomic RNA comprises at least a portion of a leader sequence. Other embodiments further comprise detecting coronavirus genomic RNA in the sample. In

some embodiments, the coronavirus is chosen from but not limited to human coronavirus 229E, human coronavirus OC43, and mouse hepatitis virus.

In addition, the present invention provides methods for inhibition of human coronavirus 229E replication comprising: i) providing a composition comprising the protease inhibitor E64D; and ii) contacting a cell permissive for 229E replication with the composition under conditions suitable for inhibiting 229E replication in the cell. In some embodiments, the composition further comprises a cyclodextrin, which in preferred embodiments is Captisol.

Furthermore, the present invention provides kits for detecting replication of a coronavirus in a sample, comprising providing: i) at least two coronavirus primers comprising a sense primer and an antisense primer; and ii) instructions for using the primers for detecting coronavirus subgenomic RNA in a sample by reverse transcriptase polymerase chain reaction (RT-PCR). Some kits further comprise providing cells susceptible for infection by a coronavirus, and instructions for using the cells for propagation of a coronavirus in a sample. In preferred embodiments, the sense primer anneals to a coronavirus leader cDNA sequence, and the antisense primer anneals to a coronavirus coding cDNA sequence. Other preferred embodiments further comprise providing a second sense primer, wherein the second sense primer anneals to a coronavirus coding cDNA sequence for simultaneously detecting coronavirus genomic RNA in a sample by RT-PCR. In some embodiments, the coronavirus is a severe acute respiratory syndrome (SARS) coronavirus, and in a subset of these the sense primer is set forth as SEQ ID NO:76, the antisense primer is set forth as SEQ ID NO:75, and the second antisense primer is set forth as SEQ ID NO:74. In further embodiments, the coronavirus is human coronavirus 229E, and in a subset of these the sense primer is set forth as SEQ ID NO:90, the antisense primer is set forth as SEQ ID NO:89, and the second antisense primer is set forth as SEQ ID NO:88. Also provided are embodiments in which the cornonavirus is human coronavirus OC43, and in a subset of these the sense primer is set forth as SEQ ID NO:96, the antisense primer is set forth as SEQ ID NO:95, and the second antisense primer is set forth as SEQ ID NO:94. In still further embodiments, the coronavirus is mouse hepatitis virus, and in a subset of these the sense primer is set forth as SEQ ID NO:93, the antisense primer is set forth as SEQ ID NO:92, and the second antisense primer is set forth as SEQ ID NO:91.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an exemplary multiplex RT-PCR assay for the detection of SARS-CoV replication. Amplification of G3PDH, SARS-CoV genomic RNA (gRNA) and subgenomic RNA (sgRNA) from RNA at 1 and 24 hours post inoculation of Vero E6 cells inoculated with serial dilutions of SARS-CoV. Mock inoculation (M). Negative images are shown. Figure is representative of 3 experiments each performed in duplicate.

Figure 2 shows susceptibility of monkey kidney cells to SARS-CoV. (A) Amplification of G3PDH, SARS-CoV gRNA and sgRNA at 1, 24, 48 h post-inoculation (p.i.). African green monkey cells (Vero E6), primary rhesus monkey kidney cells (pRhMK), primary cynomologus monkey kidney cells (pCMK). Mock inoculated cells (M) and baby hamster kidney cells (BHK21) included as negative controls. Negative images are shown. Results are representative of 2 experiments performed in duplicate. (B) Titration of cell supernatants in Vero E6 cells ($TCID_{50}$). Graph depicts the average of two to three experiments each in triplicate.

Figure 3 shows susceptibility of cells expressing known coronavirus receptors. Amplification of G3PDH, SARS-CoV gRNA and sgRNA at 1, 24 and 48 h p.i.. Human lung fibroblasts (MRC-5), canine kidney (MDCK), feline lung epithelia (AK-D), murine fibroblast (L2), and human rectal tumor (HRT-18). Vero E6 included as a positive control, mock infected as negative control (M). * denotes non-specific amplification product. Negative images are shown. Figure is representative of 2 experiments performed in duplicate.

Figure 4 shows susceptibility of clinically relevant cells to SARS-CoV. (A) Amplification of G3PDH, SARS-CoV gRNA and sgRNA at 1, 24 and 48 h p.i.. Mixed monolayer of mink lung cells and human lung cells (R-Mix), Mink lung cells (Mv1Lu), human lung cells (A549) and human embryonic lung cells (HEL). Mock inoculated cells included as negative control. Negative images are shown. Figure is representative of two experiments performed in duplicate. (B) Titration of cell supernatants in Vero E6 cells ($TCID_{50}$). Graph is average of 2 experiments performed in triplicate.

Figure 5 shows susceptibility of human cell lines to SARS-CoV. (A) Amplification of G3PDH, SARS-CoV gRNA and sgRNA at 1, 24 and 48 h p.i.. Human embryonic kidney (HEK-293T) and human liver carcinoma cells (Huh-7). Mock inoculated cells were included as a negative control (M). Negative images are shown. This Figure is representative of three experiments performed in duplicate.

Figure 6 shows the effect of human APN on susceptibility of cells. (A) Amplification of G3PDH, SARS-CoV gRNA and sgRNA at 1, 24 and 48 h p.i.. Murine epithelial cells (CMT-93), CMT-93 expressing human APN (hAPN) (CMT-93/hAPN), baby hamster kidney cells (BHK-21) and BHK-21 expressing hAPN (BHK-21/hAPN). Mock inoculated cells included as negative control; Huh-7 cells included as positive control. Negative images are shown. Figure is representative of three experiments performed in triplicate. (B) FACS analysis of APN expression. Cells transfected with hAPN are depicted by the solid line, cells without APN are depicted by the dashed line, staining with isotype control antibody is represented by the shaded curve.

Figure 7 A-I shows the genomic RNA sequence of SARS-CoV Urbani (GenBank accession # AY278741) (SEQ ID NO:1).

Figure 8 A-J shows the genomic RNA sequence of SARS-CoV Tor2 (GenBank accession # AY274119) (SEQ ID NO:2).

Figure 9 A-I shows the genomic RNA sequence of SARS-CoV CUHK-W1 (GenBank accession # AY278554) (SEQ ID NO:3).

Figure 10 shows a partial genomic RNA sequence of SARS-CoV Shanghai LY (GenBank accession # H012999) (SEQ ID NO:4) orf1a polyprotein gene.

Figure 11 A-C shows a partial genomic RNA sequence of SARS-CoV Shanghai LY (GenBank accession # H012999) (SEQ ID NO:5) orf1ab polyprotein and orf1a polyprotein genes.

Figure 12 A-B shows a partial genomic RNA sequence of SARS-CoV Shanghai LY (GenBank accession # H012999) (SEQ ID NO:6) orf1ab polyprotein, Spike glycoprotein, and Orf3a genes.

Figure 13 shows a partial genomic RNA sequence of SARS-CoV Shanhgai LY (GenBank accession # H012999) (SEQ ID NO:7) Orf7a, Orf7b, Orf8A, Orf8b, and Nucleocapsid protein genes.

Figure 14 A-B shows a partial genomic RNA sequence of SARS-CoV Shanghai QXC (GenBank accession # AH013000) (SEQ ID NO:8) orf1a polyprotein, and orf1ab polyprotein genes.

Figure 15 A-B shows a partial genomic RNA sequence of SARS-CoV Shanghai QXC (GenBank accession # AH013000) (SEQ ID NO:9) orf1ab polyprotein gene.

Figure 16 shows a partial genomic RNA sequence of SARS-CoV Shanghai QXC (GenBank accession # AH013000) (SEQ ID NO:10) of the Orf3a, Orf4b, envelope protein E, membrane glycoprotein M, Orf6, and Orf7a genes.

Figure 17 shows a partial genomic RNA sequence of SARS-CoV Shanghai LY (GenBank accession # AY322208) (SEQ ID NO:11) of the Orf7a gene (partial cds); and Orf7b, Orf8A, Orf8b, and Nucleocapsid protein genes (complete cds).

Figure 18 A-B shows a genomic RNA sequence of SARS-CoV Shanghai QXC (GenBank accession # AY322197) (SEQ ID NO:12) orf1ab polyprotein and orf1a polyprotein genes.

Figure 19 shows a genomic RNA sequence of SARS-CoV Shanghai QXC (GenBank accession # AY322199) (SEQ ID NO:13) Orf3a gene (partial cds), Orf4b, envelope protein E, membrane glycoprotein M, and Orf6 genes (complete cds), and Orf7a gene (partial cds).

Figure 20 shows a genomic RNA sequence of SARS-CoV Shanghai LY (GenBank accession # AY322205) (SEQ ID NO:14) orf1ab polyprotein and orf1a polyprotein genes (partial cds).

Figure 21 A-D shows a genomic RNA sequence of SARS-CoV Shanghai LY (GenBank accession # AY322206) (SEQ ID NO:15) orf1a polyprotein and orf1ab polyprotein genes (partial cds).

Figure 22 shows a genomic RNA sequence of SARS-CoV ZJ-HZ01 (GenBank accession # AY322206) (SEQ ID NO:16) Nucleocapsid protein, uncharacterized protein 9b, and uncharacterized protein 9c genes, (complete cds).

Figure 23 shows the amino acid sequence (SEQ ID NO:17) of Nucleocapsid protein of SARS-CoV (Urbani) (GenBank Accession Number AY278741).

Figure 24 shows the amino acid sequence (SEQ ID NO:18) of Nucleocapsid protein of SARS-CoV (Tor2) (Genbank Accession Number AY274119).

Figure 25 shows the amino acid sequence of Nucleocapsid protein of SARS-CoV (Shanghai LY) (A) for Genbank Accession Number AY322205 (SEQ ID NO:19), and (B) for Genbank Accession Number AY322208 (SEQ ID NO:80).

Figure 26 shows the amino acid sequence (SEQ ID NO:20) of Nucleocapsid protein of SARS-CoV (ZJ-HZ01) (Genbank Accession Number AY290752).

Figure 27 shows the amino acid sequence (SEQ ID NO:21) of the Spike glycoprotein of SARS-CoV (Urbani) (Genbank Accession Number AY278741).

Figure 28 shows the amino acid sequence (SEQ ID NO:22) of the Spike glycoprotein of SARS-CoV (Tor2) (Genbank Accession Number AY274119).

Figure 29 shows the amino acid sequence (SEQ ID NO:23) of the Spike glycoprotein of SARS-CoV (Shanghai LY) (Genbank Accession Number AY322205).

Figure 30 shows the amino acid sequence of the Matrix protein of SARS-CoV (A) Urbani (Genbank Accession Number AY278741) (SEQ ID NO:24), (B) Tor2 (Genbank Accession Number AY274119) (SEQ ID NO:81), (C and D) Shanghai QXC (Genbank Accession Numbers AY322199 and AH013000) (SEQ ID NO:82 and SEQ ID NO:83, respectively).

Figure 31 shows the amino acid sequence of the E protein of SARS-CoV (A) Urbani (Genbank Accession Number AY278741) (SEQ ID NO:25), (B) Tor2 (Genbank Accession Number AY274119) (SEQ ID NO:84), (C and D) Shanghai QXC (Genbank Accession Numbers AY322199 and AH013000) (SEQ ID NO:85 and SEQ ID NO:86, respectively), and (E) UHK-W1 (Genbank Accession Number AY278554) (SEQ ID NO:87).

Figure 32 A-C shows the amino acid sequence (SEQ ID NO:26) of the polyprotein 1a of SARS-CoV Urbani (Genbank Accession Number AY278741).

Figure 33 A-E shows the amino acid sequence (SEQ ID NO:27) of the polyprotein 1ab of SARS-CoV (Tor2) (Genbank Accession Number AY274119).

Figure 34 A-B shows the amino acid sequence (SEQ ID NO:28) of the polyprotein 1b of SARS-CoV (Urbani) (Genbank Accession Number AY278741).

Figure 35 A-E shows the amino acid sequence (SEQ ID NO:29) of the polyprotein 1ab of SARS-CoV (CUHK-W1) (Genbank Accession Number AY278554).

Figure 36 shows the amino acid sequence (SEQ ID NO:30) of the polyprotein 1a of SARS-CoV (Shanghai QXC) (Genbank Accession Number AY322197).

Figure 37 shows the amino acid sequence (SEQ ID NO:31) of the polyprotein 1ab of SARS-CoV (Shanghai QXC) (Genbank Accession Number AY322197).

Figure 38 shows the amino acid sequence (SEQ ID NO:32) of the polyprotein 1a of SARS-CoV (Shanghai LY) (Genbank Accession Number AY322197).

Figure 39 shows the amino acid sequence (SEQ ID NO:33) of the polyprotein 1ab of SARS-CoV (Shanghai LY) (Genbank Accession Number AY322197).

Figure 40 shows the amino acid sequence (SEQ ID NO:34) of Nucleocapsid protein of SARS-CoV (CUHK-W1) (Genbank Accession Number AY278554).

Figure 41 shows the amino acid sequence (SEQ ID NO:35) of Spike protein of SARS-CoV (CUHK-W1) (Genbank Accession Number AY278554).

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "cell type," refers to any cell, regardless of its source or characteristics. A cell type includes a "wild-type cell" (*i.e.*, a cell whose genome has not been manipulated by man), and a "transgenic cell."

As used herein, the term "microorganism" refers to any organism of microscopic or ultramicroscopic size including, but not limited to, viruses, bacteria, and protozoa.

As used herein, the term "culture" refers to a composition, whether liquid, gel, or solid, which contains one or more microorganisms and/or one or more cells. A culture of organisms and/or cells can be pure or mixed. For example, the terms "pure culture" of a microorganism as used herein refers to a culture in which the microorganisms present are of only one strain of a single species of a particular genus. This is in contrast to a "mixed culture" of microorganisms, which refers to a culture in which more than one strain of a single genus and/or species of microorganism is present.

As used herein, the terms "culture media," and "cell culture media," refer to media that are suitable to support maintenance and/or growth of cells *in vitro* (*i.e.*, cell cultures).

A "primary cell" is a cell, which is directly obtained from a tissue or organ of an animal whether or not the cell is in culture.

A "cultured cell" is a cell, which has been maintained and/or propagated *in vitro*. Cultured cells include primary cultured cells and cell lines.

"Primary cultured cells" are primary cells which are in *in vitro* culture and which preferably, though not necessarily, are capable of undergoing ten or fewer passages in *in vitro* culture before senescence and/or cessation of proliferation.

The terms "cell line" and "immortalized cell" refer to a cell, which is capable of a greater number of cell divisions *in vitro* before cessation of proliferation and/or senescence as compared to a primary cell from the same source. A cell line includes, but does not require, that the cells be capable of an infinite number of cell divisions in culture. The number of cell divisions may be determined by the number of times a cell population may be passaged (*i.e.*, subcultured) in *in vitro* culture. Passaging of cells is accomplished by methods known in the art. Briefly, a confluent or subconfluent population of cells which is

adhered to a solid substrate (*e.g.*, plastic Petri dish) is released from the substrate (*e.g.*, by enzymatic digestion), and a proportion (*e.g.*, 10%) of the released cells is seeded onto a fresh substrate. The cells are allowed to adhere to the substrate, and to proliferate in the presence of appropriate culture medium. The ability of adhered cells to proliferate may be determined visually by observing increased coverage of the solid substrate over a period of time by the adhered cells. Alternatively, proliferation of adhered cells may be determined by maintaining the initially adhered cells on the solid support over a period of time, removing and counting the adhered cells and observing an increase in the number of maintained adhered cells as compared to the number of initially adhered cells.

Cell lines may be generated spontaneously or by transformation. A "spontaneous cell line" is a cell line, which arises during routine culture of cells. A "transformed cell line" refers to a cell line that is generated by the introduction of a "transgene" comprising nucleic acid (usually DNA) into a primary cell or into a finite cell line by way of human intervention

Cell lines include, but are not limited to, finite cell lines and continuous cell lines. As used herein, the term "finite cell line" refers to a cell line, which is capable of a limited number (from about 1 to about 50, more preferably from about 1 to about 40, and most preferably from about 1 to about 20) of cell divisions prior to senescence.

The term "continuous cell line" refers to a cell line, which is capable of more than about 50 (and more preferably, an infinite number of) cell divisions. A continuous cell line generally, although not necessarily, also has the general characteristics of a reduced cell size, higher growth rate, higher cloning efficiency, increased tumorigenicity, and/or a variable chromosomal complement as compared to the finite cell line or primary cultured cells from which it is derived.

The term "transgene" as used herein refers to any nucleic acid sequence, which is introduced into the cell by experimental manipulations. A transgene may be an "endogenous DNA sequence" or a "heterologous DNA sequence" (*i.e.*, "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence, which is naturally found in the cell into which it is introduced so long as it does not contain some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, *etc.*) relative to the naturally-occurring sequence. The term "heterologous DNA sequence" refers to a nucleotide sequence, which is ligated to, or is manipulated for ligation to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location

in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Heterologous DNA also includes an endogenous DNA sequence, which contains some modification. Generally, although not necessarily, heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is expressed. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, selectable marker proteins (*e.g.*, proteins which confer drug resistance), *etc.*

The term "nucleotide sequence of interest" refers to any nucleotide sequence, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art. Nucleotide sequences of interest include, but are not limited to, coding sequences of structural genes (*e.g.*, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*), and non-coding regulatory sequences which do not encode an mRNA or protein product, (*e.g.*, promoter sequence, polyadenylation sequence, termination sequence, enhancer sequence, *etc.*).

As used herein, the singular forms "a," "an" and "the" include both singular and plural references unless the content clearly dictates otherwise.

As used herein, the term "or" when used in the expression "A or B," where A and B refer to a composition, disease, product, *etc.*, means one, or the other, or both.

The terms "chosen from A, B and C" and "chosen from one or more of A, B and C" are equivalent terms that mean selecting any one of A, B, and C, or any combination of A, B, and C.

As used herein, the term "comprising" when placed before the recitation of steps in a method means that the method encompasses one or more steps that are additional to those expressly recited, and that the additional one or more steps may be performed before, between, and/or after the recited steps. For example, a method comprising steps a, b, and c encompasses a method of steps a, b, x, and c, a method of steps a, b, c, and x, as well as a method of steps x, a, b, and c. Furthermore, the term "comprising" when placed before the recitation of steps in a method does not (although it may) require sequential performance of the listed steps, unless the content clearly dictates otherwise. For example, a method comprising steps a, b, and c encompasses, for example, a method of performing steps in the order of steps a, c, and b, the order of steps c, b, and a, and the order of steps c, a, and b, *etc.*

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth as used herein, are to

be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters herein are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and without limiting the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

The term "not" when preceding, and made in reference to, any particularly named molecule (*e.g.*, nucleic acid sequence such as "gRNA," "sgRNA," amino acid sequence such as "Nucleocapsid," "Spike," "Matrix," "E protein," and "Replicase proteins," *etc.*), and/or phenomenon (*e.g.*, susceptibility, permissivity, infection with a microorganism, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) means that only the particularly named molecule or phenomenon is excluded.

The term "altering" and grammatical equivalents as used herein in reference to the level of any molecule (*e.g.*, nucleic acid sequence such as "gRNA," "sgRNA," amino acid sequence such as "Nucleocapsid," "Spike," "Matrix," "E protein," and "Replicase proteins," *etc.*), and/or phenomenon (*e.g.*, susceptibility, permissivity, infection with a microorganism, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) refers to an increase and/or decrease in the quantity of the molecule and/or phenomenon, regardless of whether the quantity is determined objectively, and/or subjectively. For example, "altering replication" of a virus includes increasing and/or decreasing the quantity of any one or more of the steps of adsorption (*e.g.*, receptor binding) to a cell, entry into a cell (such as by endocytosis), introducing the viral genome sequence into the cell, uncoating the viral genome, initiating transcription of genomic RNA, producing subgenomic RNA, directing expression of SARS-CoV encapsidation proteins, encapsidating the replicated viral nucleic acid sequence with the encapsidation proteins into a viral particle, release of the encapsidated virus from the cell, and infection of other cells by the released virus.

Unless defined otherwise in reference to the level of molecules and/or phenomena, the terms "increase," "elevate," "raise," and grammatical equivalents when in reference to the level of any molecule (*e.g.*, nucleic acid sequence such as "gRNA," "sgRNA," amino acid sequence such as "Nucleocapsid," "Spike," "Matrix," "E protein," and "Replicase proteins," *etc.*), and/or phenomenon (*e.g.*, susceptibility, permissivity, infection with a

microorganism, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) in a first sample relative to a second sample, mean that the quantity of the molecule and/or phenomenon in the first sample is higher than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the increase may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, difficulty in breathing, clarity of vision, nausea, tiredness, *etc.* In another embodiment, the quantity of the molecule and/or phenomenon in the first sample is at least 10% greater than, at least 25% greater than, at least 50% greater than, at least 75% greater than, and/or at least 90% greater than the quantity of the same molecule and/or phenomenon in a second sample.

Unless defined otherwise in reference to the level of molecules and/or phenomena, the terms "reduce," "inhibit," "diminish," "suppress," "decrease," and grammatical equivalents when in reference to the level of any molecule (*e.g.*, nucleic acid sequence such as "gRNA," "sgRNA," amino acid sequence such as "Nucleocapsid," "Spike," "Matrix," "E protein," and "Replicase proteins," *etc.*), and/or phenomenon (*e.g.*, susceptibility, permissivity, infection with a microorganism, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) in a first sample relative to a second sample, mean that the quantity of molecule and/or phenomenon in the first sample is lower than in the second sample by the specified amount. In one embodiment, the reduction may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, difficulty in breathing, clarity of vision, nausea, tiredness, *etc.* In another embodiment, the quantity of molecule and/or phenomenon in the first sample is at least 10% lower than, at least 25% lower than, at least 50% lower than, at least 75% lower than, and/or at least 90% lower than the quantity of the same molecule and/or phenomenon in a second sample.

Reference herein to any specifically named protein (such as "Nucleocapsid," "Spike," "Matrix," "E protein," and "Replicase proteins," *etc.*) refers to any and all equivalent fragments, fusion proteins, and variants of the specifically named protein, having at least one of the biological activities (such as those disclosed herein and/or known in the art) of the specifically named protein, wherein the biological activity is detectable by any method.

The term "fragment" when in reference to a protein (such as "Nucleocapsid," "Spike," "Matrix," "E protein," and "Replicase proteins," *etc.*) refers to a portion of that protein that may range in size from four (4) contiguous amino acid residues to the entire amino acid sequence minus one amino acid residue. Thus, a polypeptide sequence comprising "at least a portion of an amino acid sequence" comprises from four (4) contiguous amino acid residues of the amino acid sequence to the entire amino acid sequence.

The term "fusion protein" refers to two or more polypeptides that are operably linked. The term "operably linked" when in reference to the relationship between nucleic acid sequences and/or amino acid sequences refers to linking the sequences such that they perform their intended function. For example, operably linking a promoter sequence to a nucleotide sequence of interest refers to linking the promoter sequence and the nucleotide sequence of interest in a manner such that the promoter sequence is capable of directing the transcription of the nucleotide sequence of interest and/or the synthesis of a polypeptide encoded by the nucleotide sequence of interest. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "variant" of a protein (such as "Nucleocapsid," "Spike," "Matrix," "E protein," and "Replicase proteins," *etc.*) as used herein is defined as an amino acid sequence, which differs by insertion, deletion, and/or conservative substitution of one or more amino acids from the protein of which it is a variant. The term "conservative substitution" of an amino acid refers to the replacement of that amino acid with another amino acid, which has a similar hydrophobicity, polarity, and/or structure. For example, the following aliphatic amino acids with neutral side chains may be conservatively substituted one for the other: glycine, alanine, valine, leucine, isoleucine, serine, and threonine. Aromatic amino acids with neutral side chains, which may be conservatively substituted one for the other include phenylalanine, tyrosine, and tryptophan. Cysteine and methionine are sulphur-containing amino acids, which may be conservatively substituted one for the other. Also, asparagine may be conservatively substituted for glutamine, and *vice versa*, since both amino acids are amides of dicarboxylic amino acids. In addition, aspartic acid (aspartate) may be conservatively substituted for glutamic acid (glutamate) as both are acidic, charged (hydrophilic) amino acids. Also, lysine, arginine, and histidine may be conservatively substituted one for the other since each is a basic, charged (hydrophilic) amino acid. Guidance in determining which and how many amino acid residues may be

substituted, inserted or deleted without abolishing biological and/or immunological activity may be found using computer programs well known in the art, for example, DNAStar™ software. In one embodiment, the sequence of the variant has at least 95% identity, at least 90% identity, at least 85% identity, at least 80% identity, at least 75% identity, at least 70% identity, and/or at least 65% identity with the sequence of the protein in issue.

Reference herein to any specifically named nucleotide sequence (such as a sequence encoding "Nucleocapsid," "Spike," "Matrix," "E protein," and "Replicase proteins," *etc.*) includes within its scope any and all equivalent fragments, homologs, and sequences that hybridize under highly stringent and/or medium stringent conditions to the specifically named nucleotide sequence, and that have at least one of the biological activities (such as those disclosed herein and/or known in the art) of the specifically named nucleotide sequence, wherein the biological activity is detectable by any method.

The "fragment" or "portion" may range in size from an exemplary 5, 10, 20, 50, or 100 contiguous nucleotide residues to the entire nucleic acid sequence minus one nucleic acid residue. Thus, a nucleic acid sequence comprising "at least a portion of" a nucleotide sequence comprises from five (5) contiguous nucleotide residues of the nucleotide sequence to the entire nucleotide sequence.

The term "homolog" of a specifically named nucleotide sequence refers to an oligonucleotide sequence, which exhibits greater than 50% identity to the specifically named nucleotide sequence. Alternatively, or in addition, a homolog of a specifically named nucleotide sequence is defined as an oligonucleotide sequence which has at least 95% identity, at least 90% identity, at least 85% identity, at least 80% identity, at least 75% identity, at least 70% identity, and/or at least 65% identity to nucleotide sequence in issue.

With respect to sequences that hybridize under stringent conditions to the specifically named nucleotide sequence, high stringency conditions comprise conditions equivalent to binding or hybridization at 68°C in a solution containing 5X SSPE, 1% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution containing 0.1X SSPE, and 0.1% SDS at 68°C. "Medium stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH_2PO_4-H_2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 1.0X SSPE, 1.0% SDS at 42°C.

The term "equivalent" when made in reference to a hybridization condition as it relates to a hybridization condition of interest means that the hybridization condition and the hybridization condition of interest result in hybridization of nucleic acid sequences which have the same range of percent (%) homology. For example, if a hybridization condition of interest results in hybridization of a first nucleic acid sequence with other nucleic acid sequences that have from 85% to 95% homology to the first nucleic acid sequence, then another hybridization condition is said to be equivalent to the hybridization condition of interest if this other hybridization condition also results in hybridization of the first nucleic acid sequence with the other nucleic acid sequences that have from 85% to 95% homology to the first nucleic acid sequence.

As will be understood by those of skill in the art, it may be advantageous to produce a nucleotide sequence encoding a protein of interest, wherein the nucleotide sequence possesses non-naturally occurring codons. Therefore, in some embodiments, codons preferred by a particular prokaryotic or eukaryotic host (Murray et al., Nucl. Acids Res., 17 (1989)) are selected, for example, to increase the rate of expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

The term "naturally occurring" as used herein when applied to an object (such as cell, tissue, *etc.*) and/or molecule (such as amino acid, amino acid sequence, nucleic acid, nucleic acid sequence, codon, *etc.*) means that the object and/or molecule can be found in nature. For example, a naturally occurring polypeptide sequence refers to a polypeptide sequence that is present in an organism (including viruses) that can be isolated from a source in nature, wherein the polypeptide sequence has not been intentionally modified in the laboratory.

A "composition" comprising a particular polynucleotide sequence and/or comprising a particular protein sequence as used herein refers broadly to any composition containing the recited polynucleotide sequence (and/or its equivalent fragments, homologs, and sequences that hybridize under highly stringent and/or medium stringent conditions to the specifically named nucleotide sequence) and/or the recited protein sequence (and/or its equivalent fragments, fusion proteins, and variants), respectively. The composition may comprise an aqueous solution containing, for example, salts (*e.g.*, NaCl), detergents (*e.g.*, SDS), and other components (*e.g.*, Denhardt's solution, dry milk, salmon sperm DNA, etc.).

The terms nucleotide sequence "comprising a particular nucleic acid sequence" and protein "comprising a particular amino acid sequence" and equivalents of these terms, refer to any nucleotide sequence of interest and to any protein of interest, respectively, that contain the particularly named nucleic acid sequence (and/or its equivalent fragments, homologs, and sequences that hybridize under highly stringent and/or medium stringent conditions to the specifically named nucleotide sequence) and the particularly named amino acid sequence (and/or its equivalent fragments, fusion proteins, and variants), respectively. The invention does not limit the source (*e.g.*, cell type, tissue, animal, *etc.*), nature (*e.g.*, synthetic, recombinant, purified from cell extract, *etc.*), and/or sequence of the nucleotide sequence of interest and/or protein of interest. In one embodiment, the nucleotide sequence of interest and protein of interest include coding sequences of structural genes (*e.g.*, probe genes, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*).

DESCRIPTION OF THE INVENTION

The invention provides compositions and methods for detecting the presence of SARS-coronavirus, and screening anti-SARS-coronavirus drugs and vaccines. These compositions and methods were premised, at least in part, on the inventors' discovery of a sensitive assay for determining susceptibility and/or permissivity of cells to SARS-CoV. The invention's compositions and methods are useful for culturing SARS-CoV isolates, producing SARS-CoV virions and/or antigens that may be used in vaccine formulations, as antigen preparations for diagnostic applications, and for screening antiviral drugs. Additional uses of the invention's compositions and methods may be found in the elucidation of potential animal models and the identification of the SARS-CoV receptor(s).

Also provided are compositions and methods for reducing infection with plus-strand RNA viruses such as SARS-coronavirus. In one embodiment, the invention provides compositions and methods for reducing infection with SARS-coronavirus, without substantially reducing infection with other respiratory viruses. These methods are premised, at least in part, on the inventors' discovery that protease inhibitors do not substantially reduce infection of cells by the exemplary respiratory viruses influenza, parainfluenza, RSV, and adenovirus (Example 8). This is in contrast to the inhibition in replication of coronaviruses by the protease inhibitor E64 (Example 8) and the cysteine proteinase inhibitor (2S,3S)transepoxysuccinyl-*L*-leucylamido-3-methylbutane ethyl ester

(Yount et al. PNAS 100:12995-13000 (2003). These methods are useful, where it is desirable to reduce exposure of personnel to SARS-coronavirus in clinical virology laboratories that routinely screen clinical specimen for respiratory pathogens (such as influenza, parainfluenza, RSV, and adenovirus) other than SARS-coronavirus.

In one embodiment, the invention's methods for detecting SARS-coronavirus are exemplified by a multiplex RT-PCR assay for detecting G3PDH, SARS-CoV genomic RNA (gRNA) and subgenomic RNA (sgRNA). In one embodiment, subgenomic RNA is indicative of virus entry and replication. The sensitivity of the PCR assay was determined by inoculation of Vero E6 cells with serial dilutions of SARS-CoV. Human, murine, canine, hamster, feline, mink and monkey cells were analyzed at various times post-inoculation and supernatants were titered to determine if cells produced infectious virus.

The invention is further premised on the discovery, using the exemplary multiplex RT-PCR assay, of mink, human, and monkey cells that are permissive to SARS-CoV infection. In particular, kidney cells derived from different species of monkey primary Rhesus (*Macaca mulatta*) monkey kidney cells (pRhMK) and primary Cynomolgus (*Macaca fascicularis*) monkey kidney cells (pCMK) were discovered by the inventors to be susceptible to, and to be productively infected by SARS-CoV. Similarly, the inventors contemplate that other primary kidney cells of other *Macaca sp.* are also susceptible to SARS-CoV infection. Data herein also shows that mink lung (Mv1Lu) epithelial cells are also susceptible to, and productively infected by SARS-CoV. In addition, the data shows that SARS-CoV does not use the receptor for serogroup 1 coronaviruses (APN/CD13) or the receptor for murine coronavirus (CEACAM 1a).

The invention is further described under (A) Coronaviruses, (B) Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), (C) Cytopathic Effect Does Not Always Correlate With SARS-CoV infection, (D) SARS-CoV Does Not Bind to The Group 1 Coronavirus Receptor aminopeptidase N (APN/CD13) and The Group 2 Coronavirus Receptor carcinoembryonic antigen (CEACAM1a), (E) Cells Permissive To SARS-CoV, (F) Exemplary Assays For Detecting Replication Of SARS-CoV, (G) Detecting Replication of SARS-CoV Using The Invention's Exemplary Cells, (H) Screening Anti-SARS-CoV Agents, (I) Administering Anti-SARS-CoV Agents, (J) Producing SARS-CoV And SARS-CoV polypeptides, and (K) Compositions And Methods For Using Protease Inhibitors To Reduce SARS-CoV Infection. Additional details are found in Gillim-Ross et al., J Clin Microbiol, 42:3196-3206, 2004, herein incorporated by reference in its entirety.

A. Coronaviruses

Coronaviruses (order *Nidovirales*, family *Coronaviridae*) are a diverse group of enveloped, positive-stranded RNA viruses. The coronavirus genome, approximately 27-32 Kb in length, is the largest found in any of the RNA viruses. Large Spike (S) glycoproteins protrude from the virus particle giving coronaviruses a distinctive corona-like appearance when visualized by electron microscopy. Coronaviruses infect a wide variety of species, including canine, feline, porcine, murine, bovine, avian and human (Holmes, et al., 1996. Coronaviridae: the viruses and their replication, p. 1075-1094. *In* Fields (ed.), Fields Virology. Lippincott-Raven, Philadelphia, PA). However, the natural host range of each coronavirus strain is narrow, typically consisting of a single species.

Coronaviruses typically bind to target cells through Spike-receptor interactions and enter cells by receptor mediated endocytosis or fusion with the plasma membrane (Holmes, et al., 1996, *supra*). The Spike-receptor interaction is a strong determinant of species specificity as demonstrated for both group 1 and group 2 coronaviruses. The receptor for group 1 coronaviruses, including human coronavirus 229E (HCoV-229E), feline coronavirus (FCoV) and porcine coronavirus (PCoV) has been identified as aminopeptidase N (APN/CD13) (Delmas, et al., 1992, Nature 357:417-420; Tresnan, et al., 1996, J.Virol. 70:8669-8674; Yeager, et al., 1992, Nature 357:420-422). APN/CD13 is a 150- to 160-kDa type II protein that is a membrane peptidase (Look, et al., 1989, J.Clin.Invest 83:1299-1307). Expression of cDNAs encoding APN in cells from species normally resistant to infection, renders them susceptible to infection (Delmas, et al., 1992, Nature 357:417-420; Yeager, et al., 1992, Nature 357:420-422). APN is typically used in a species-specific manner (*e.g.,* PCoV binds porcine APN, HCoV-229E binds hAPN, etc.) (Benbacer, et al., J.Virol. 71:734-737; Kolb, et al., 1997. J.Gen.Virol. 78 (Pt 11):2795-2802; Wentworth, et al., 2001, J.Virol. 75:9741-9752). However, feline APN acts as a universal receptor for group 1 coronaviruses (Tresnan, et al., 1996, J.Virol. 70:8669-8674).

The receptor used by MHV, a group 2 coronavirus, was identified as a biliary glycoprotein in the carcinoembryonic antigen (CEA) family of the immunoglobulin superfamily (CEACAM) (Williams, et al., 1991, Proc.Natl.Acad.Sci.U.S.A 88:5533-5536; Williams, et al., 1990, J.Virol. 64:3817-3823). MHV binds a mouse-specific epitope of CEACAM known as CEACAM1a, and it is this species specificity of virus binding that is believed to be a principal determinant of the restricted host range of MHV infection (Compton, et al., 1982, J.Virol. 66:7420-7428). CEACAM1a cDNA transfected into MHV

resistant cell lines renders the cells susceptible to infection with MHV-A59 and MHV-JHM (Dveksler, et al., 1996, J.Virol. 70:4142-4145; Dveksler, et al., 1991, J.Virol. 65:6881-6891). Additionally, SL/J mice, which express an allelic variant of CEACAM1a, are resistant to MHV-A59 (Dveksler, et al., 1995, J.Virol. 69:543-546).

Upon entry into susceptible cells, the open reading frame (ORF) nearest the 5' terminus of the coronavirus genome is translated into a large polyprotein. This polyprotein is autocatalytically cleaved by viral-encoded proteases, to yield multiple proteins that together serve as a virus-specific, RNA-dependent RNA polymerase (RdRP). The RdRP replicates the viral genome and generates 3' coterminal nested subgenomic RNAs. Subgenomic RNAs include capped, polyadenylated RNAs that serve as mRNAs, and antisense subgenomic RNAs complementary to mRNAs. In one embodiment, each of the subgenomic RNA molecules shares the same short leader sequence fused to the body of each gene at conserved sequence elements known as intergenic sequences (IGS), transcriptional regulating sequences (TRS) or transcription activation sequences. It has been controversial as to whether the nested subgenomic RNAs are generated during positive or negative strand synthesis; however, recent work favors the model of discontinuous transcription during minus strand synthesis (Sawicki, et al., 1995, Adv.Exp.Med.Biol. 380:499-506; Sawicki and Sawicki Adv. Expt. Biol. 1998, 440:215).

B. Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)

The terms "SARS coronavirus," "SARS-CoV," and "severe acute respiratory syndrome coronavirus" are equivalent, and are used to refer to an RNA virus that is the causative agent of severe acute respiratory syndrome (Drosten, et al., 2003, *supra*; Fouchier, et al., 2003, *supra*; Ksiazek, et al., 2003, *supra*; Peiris, et al., 2003, , *supra*; Poutanen, et al., 2003, *supra*). Exemplary strains of SARS coronavirus include, but are not limited to, Urbani, Tor2, CUHK-W1, Shanghai LY, Shanghai QXC, ZJ-HZ01, TW1, HSR 1, WHU, TWY, TWS, TWK, TWJ, TWH, HKU-39849, FRA, TWC3, TWC2, TWC, ZMY 1, BJ03, ZJ01, CUHK-Su10, GZ50, SZ16, SZ3, CUHK-W1, BJ04, AS, Sin2774, GD01, Sin2500, Sin2677, Sin2679, Sin2748, ZJ-HZ01, and BJ01. While the invention is illustrated using SARS-CoV from humans, the term "SARS coronavirus" expressly includes within its scope equivalent coronaviruses that cause equivalent severe acute respiratory syndromes in other mammals (such as, without limitation, monkey, hamster, mink, ferret, pig, cat, and rabbit), insects (such as mosquito), *etc.*

The genome of SARS-CoV contains a single stranded (+)-sense RNA. Complete and partial genome sequences of several SARS coronavirus isolates have been reported, including SARS coronavirus Urbani (GenBank accession # AY278741, Figure 7), SARS coronavirus Tor2 (GenBank accession # AY274119, Figure 8), SARS coronavirus CUHK-W1 (GenBank accession # AY278554, Figure 9), SARS-CoV Shanhgai LY (GenBank accession # H012999, Figures 10-13; GenBank accession # AY322205, Figure 20; GenBank accession # AY322206, Figure 21), SARS-CoV Shanghai QXC (GenBank accession # AH013000, Figures 14-16; GenBank accession # AY322208, Figure 17; GenBank accession # AY322197, Figure 18; GenBank accession # AY322199, Figure 19), and SARS-CoV ZJ-HZ01 (GenBank accession # AY322206, Figure 22), gi|31416292|gb|AY278487.3| SARS coronavirus BJ02,gi|30248028|gb|AY274119.3| SARS coronavirus TOR2, gi|30698326|gb|AY291451.1| SARS coronavirus TW1, gi|33115118|gb|AY323977.2| SARS coronavirus HSR 1, gi|35396382|gb|AY394850.1| SARS coronavirus WHU, gi|33411459|dbj|AP006561.1| SARS coronavirus TWY, gi|33411444|dbj|AP006560.1| SARS coronavirus TWS, gi|33411429|dbj|AP006559.1| SARS coronavirus TWK, gi|33411414|dbj|AP006558.1| SARS coronavirus TWJ, gi|33411399|dbj|AP006557.1| SARS coronavirus TWH, gi|30023963|gb|AY278491.2| SARS coronavirus HKU-39849, gi|33578015|gb|AY310120.1| SARS coronavirus FRA, gi|33518725|gb|AY362699.1| SARS coronavirus TWC3, gi|33518724|gb|AY362698.1| SARS coronavirus TWC2, gi|30027617|gb|AY278741.1| SARS coronavirus Urbani, gi|31873092|gb|AY321118.1| SARS coronavirus TWC, gi|33304219|gb|AY351680.1| SARS coronavirus ZMY 1, gi|31416305|gb|AY278490.3| SARS coronavirus BJ03, gi|30910859|gb|AY297028.1| SARS coronavirus ZJ01, gi|30421451|gb|AY282752.1| SARS coronavirus CUHK-Su10, gi|34482146|gb|AY304495.1| SARS coronavirus GZ50, gi|34482139|gb|AY304488.1| SARS coronavirus SZ16, gi|34482137|gb|AY304486.1| SARS coronavirus SZ3, gi|30027610|gb|AY278554.2| SARS coronavirus CUHK-W1, gi|31416306|gb|AY279354.2| SARS coronavirus BJ04, gi|37576845|gb|AY427439.1| SARS coronavirus AS, gi|37361915|gb|AY283798.2| SARS coronavirus Sin2774, gi|31416290|gb|AY278489.2| SARS coronavirus GD01, gi|30468042|gb|AY283794.1| SARS coronavirus Sin2500, gi|30468043|gb|AY283795.1| SARS coronavirus Sin2677, gi|30468044|gb|AY283796.1| SARS coronavirus Sin2679, gi|30468045|gb|AY283797.1| SARS coronavirus Sin2748, gi|31982987|gb|AY286320.2| SARS coronavirus isolate ZJ-HZ01, gi|30275666|gb|AY278488.2| SARS coronavirus BJ01.

SARS-CoV may be productive or replication defective. A "productive" SARS-CoV refers to a SARS-CoV particle that is capable of replication. The term "replication" includes, but is not limited to, the steps of adsorbing (*e.g.*, receptor binding) to a cell, entry into a cell (such as by endocytosis), introducing its genome sequence into the cell, uncoating the viral genome, initiating transcription of SARS-CoV genomic RNA to produce sgRNA, directing expression of SARS-CoV encapsidation proteins, encapsidating of the replicated viral nucleic acid sequence with the encapsidation proteins into a viral particle that is released from the cell to infect other cells that are of either a permissive or susceptible character. The terms "replication defective," "replication-incompetent," and "defective" SARS-CoV refer to a SARS-CoV particle, which is substantially incapable of one or more of the steps of replication.

The origin of SARS-CoV has not been determined but its emergence may be the result of zoonotic transmission. The location and source of the SARS-CoV outbreak are reminiscent of influenza pandemics that have killed millions of people in the past.

SARS-CoV has been isolated from humans, civet cats and a raccoon-dog, and has been propagated in kidney cells derived from different species of monkey (Drosten, et al., 2003, N.Engl.J.Med. 348:1967-1976; Ksiazek, et al., 2003, N.Engl.J.Med. 348:1953-1966; Peiris, et al., 2003, Lancet 361:1319-1325; Poutanen, et al., 2003, N.Engl.J.Med. 348:1995-2005). Coronaviruses typically demonstrate a narrow host range and are species specific yet, SARS-CoV appears to have a broad host range. Furthermore, while disease caused by the known humans coronaviruses is mild, SARS-CoV, like some of the animal coronaviruses, causes fatal disease (Holmes, et al., 1996, *supra*).

C. Cytopathic Effect Does Not Always Correlate With SARS-CoV infection

Cell lines are routinely utilized to screen clinical specimen for respiratory pathogens. At the beginning of the SARS-CoV outbreak, clinical specimens were inoculated onto panels of cells to identify the causative agent of SARS (Drosten, et al., 2003, N.Engl.J.Med. 348:1967-1976; Ksiazek, et al., 2003, N.Engl.J.Med. 348:1953-1966; Peiris, et al., 2003, Lancet 361:1319-1325). Based on CPE, Vero E6 and FRhMK cells were identified as susceptible to SARS-CoV infection (Drosten, et al., 2003, N.Engl.J.Med. 348:1967-1976; Ksiazek, et al., 2003, N.Engl.J.Med. 348:1953-1966; Peiris, et al., 2003, Lancet 361:1319-1325). Surprisingly, however, coronaviruses can establish persistent infection in cells without inducing CPE, suggesting that CPE may not be an accurate

indicator of infection (Chaloner, et al., 1981, Arch.Virol. 69:117-129). Data herein confirmed this surprising observation by demonstrating replication of SARS-CoV in the absence of CPE. For example, significant CPE was not observed in pRhMK or pCMK 5 days p.i., although the inventors discovered that virus titers were actually increased within 24 hours p.i. (Figure 2B, Table 1).

TABLE 1. Susceptibility of Cells to SARS-CoV

Cell	Species of Origin	SARS-CoV Replication	CPE	Viral Titer at 48hr ($TCID_{50}$)
Vero E6	African green monkey kidney	+	+	$2.4x10^7$
pRhMK	1° rhesus monkey kidney	+	-	$5.6x10^5$
pCMK	1° cynomologus monkey kidney	+	-	$7.8x10^4$
MRC-5	Human lung fibroblast	-	-	0
MDCK	Canine kidney	-	-	N/D
AK-D	Feline lung epithelia	-	-	N/D
HRT-18	Human rectal tumor	-	-	0
L2	Murine fibroblast	-	-	N/D
R-Mix	Mink and Human lung	+	-	$7.8x10^3$
Mv1Lu	Mink lung	+	-	$2.5x10^4$
A549	Human lung epithelia	-	-	N/D
HEL	Human embryonic lung	-	-	0
HEK-293T	Human embryonic kidney	+	-	$5.6x10^3$
Huh-7	Human liver	+	-	$1.3x10^5$
CMT-93	Murine epithelia	-	-	N/D
CMT-93/hAPN	Murine epithelia	-	-	N/D
BHK	Baby hamster kidney	-	-	N/D
BHK/hAPN	Baby hamster kidney	-	-	N/D

D. SARS-CoV Does Not Bind to The Group 1 Coronavirus Receptor aminopeptidase N (APN/CD13) and The Group 2 Coronavirus Receptor carcinoembryonic antigen (CEACAM1a)

Aminopeptidase N, the receptor for group 1 coronaviruses is expressed on the surface of epithelial cells of the kidney. The identification of monkey kidney cells susceptible to SARS-CoV, the culturing of SARS-CoV from the kidney of an infected patient, and sequence-based studies predicting that the SARS-CoV Spike glycoprotein contains APN binding domains, led to the proposal that APN is a potential receptor for SARS-CoV (Yu, et al., 2003, Acta Pharmacol.Sin. 24:481-488). Based on these assumptions, APN inhibitors were proposed for the treatment of SARS-CoV infection (Kontoyiannis, et al., 2003, Lancet 361:1558). However, data herein (such as Examples 4 and 7) show that cells expressing species specific APN molecules as well as feline APN, the universal group 1 receptor, were all non permissive to SARS-CoV. Even cells expressing high levels of hAPN, previously demonstrated to be susceptible to HCoV-229E, were non permissive to SARS-CoV, suggesting that SARS-CoV uses a receptor other than APN (Wentworth et al. 2001. J.Virol. 75:9741-9752.). Snijder et al. has suggested that SARS-CoV is most closely related to Group II coronaviruses, suggesting that it may use a receptor utilized by a group 2 coronavirus. However, cell lines permissive to group 2 coronaviruses were not susceptible to SARS-CoV. Murine cells expressing CEACAM1a, the receptor for MHV and HRT-18 cells which are susceptible to HCoV-0C43, were both non permissive to SARS-CoV infection. The inventors' findings suggest that SARS-CoV utilizes a yet unidentified receptor.

E. Cells Permissive To SARS-CoV

SARS-CoV was first isolated in African green monkey kidney cells (Vero E6) and fetal rhesus monkey kidney cells (FRhMK) inoculated with clinical specimen (Drosten, et al., 2003, N.Engl.J.Med. 348:1967-1976; Ksiazek, et al., 2003, N.Engl.J.Med. 348:1953-1966; Peiris, et al., 2003, Lancet 361:1319-1325; Poutanen, et al., 2003, N.Engl.J.Med. 348:1995-2005). Based on cytopathic effect (CPE), other cells routinely used for identification of respiratory pathogens were determined to be non-permissive to SARS-CoV infection, such as MDCK, A549, NCI-H292, HeLa, LLC-MK2, Hut-292, B95-8, MRC-5, RDE and Hep-2 (Drosten, et al., 2003, N.Engl.J.Med. 348:1967-1976; Ksiazek, et al., 2003, N.Engl.J.Med. 348:1953-1966; Peiris, et al., 2003, Lancet 361:1319-1325).

To identify cell lines permissive to SARS-CoV, a multiplex reverse transcriptase polymerase chain reaction (RT-PCR) assay for detection of SARS-CoV replication was developed by the inventors, as described herein (Example 2). Primary cells and continuous cell lines derived from a number of species and tissues were analyzed for susceptibility to SARS-CoV. Additionally, cells routinely used by clinical virology laboratories for pathogen screening were analyzed for susceptibility to SARS-CoV. Data herein demonstrates the identification of identified both human and non-human (monkey and mink) cells that support SARS-CoV replication (Examples 3, 5, and 6, Table 1).

In particular, data herein (Example 3, Table 1) show that kidney cells derived from three different species of monkey (African green monkey, Rhesus macaque and Cynomolgus macaque) were susceptible to productive SARS-CoV infection. However, infection of pCMK and pRhMK cells resulted in lower viral titers than infection of Vero E6 cells. Without intending to limit the invention to any particular mechanism, and while an understanding of the mechanism of the invention is not required, it is the inventors' consideration that the discrepancy in virus production may be due to Vero E6 cells being a transformed cell line while pCMK and pRhMK are both primary cell populations. Furthermore, pCMK and pRhMK are both mixed cell populations; the cells susceptible to SARS-CoV may make up only a small percentage of the total cell population. Thus, in certain embodiments, it may be more advantageous to use cell lines such as HEK-293T, Huh-7 and Mv1Lu cells as compared to primary cells such as pCMK and pRhMK.

Kuiken et al. recently demonstrated that Cynomolgus Macaques inoculated with SARS-CoV develop clinical symptoms similar to those observed in infected humans. SARS-CoV was subsequently isolated from the inoculated monkeys (Fouchier, et al., 2003, Nature 423:240; Kuiken, et al., 2003, Lancet 362:263-270). However, SARS-CoV was not detected in kidney from these animals by immunohistochemical techniques. Surprisingly, therefore, and in contrast to Kuiken et al.'s report, the inventors' data suggest that kidney cells from monkeys supports SARS-CoV replication (Ksiazek, et al., 2003, N.Engl.J.Med. 348:1953-1966).

Data herein (Example 5, Table 1) also identifies mink lung cells (Mv1Lu) as susceptible to SARS-CoV productive infection. In contrast, all of the clinically relevant cells that were tested by the inventors were not susceptible to SARS-CoV infection. Mv1Lu cells are incorporated into respiratory panels, that are used to screen clinical specimen for respiratory pathogens including influenza A and B, adenovirus, RSV and

parainfluenza. Additionally, Mv1Lu cells are a component of R-Mix, the cell mix that is also used for detection of RSV and parainfluenza viruses. Without intending to limit the invention to any mechanism, and while an understanding of the mechanism of the invention is not necessary, it is the inventors' view that the low level of virus replication detected in Mv1Lu cells by titration may be due to slower replication of SARS-CoV in mink-derived cells than in Vero E6 cells where the virus was passaged.

Data herein (Example 6, Table 1) further demonstrates that two human-derived cell lines are susceptible to SARS-CoV productive infection. A kidney cell line (HEK-293T) and a liver cell line (Huh-7) were both permissive to SARS-CoV infection. HEK-293T cells were susceptible to SARS-CoV infection but do not support production of high titers of virus, suggesting that these cells may contain a block to SARS-CoV replication. Conversely, Huh-7 cells produced higher titers of SARS-CoV although the titers were still lower then those produced from infected Vero E6 cells. Without intending to limit the invention to any mechanism, and while an understanding of the mechanism of the invention is not necessary, it is the inventors' opinion that the discrepancy in viral titers may be due to the passage of the virus in Vero E6 cells where it may have adapted.

In one embodiment, the cells used in any one of the invention's methods are chosen from one or more of HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK.

While the invention is illustrated using HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK cells, it should be understood that the invention is not limited to these particular cells, but rather includes equivalent cells that are established from these particular cells.

The term "established from" when made in reference to any cell disclosed herein (such as HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) refers to a cell which has been obtained (*e.g.*, isolated, purified, *etc.*) from the parent cell in issue using any manipulation, such as, without limitation, infection with virus, transfection with DNA sequences, treatment and/or mutagenesis using for example chemicals, radiation, *etc.*, selection (such as by serial culture) of any cell that is contained in cultured parent cells. For example, the invention includes within its scope cell lines that may be established from any cell disclosed herein (such as HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) by treatment with chemical compounds (*e.g.*, *N*-ethyl-*N*-nitrosurea (ENU), methylnitrosourea (MNU), procarbazine hydrochloride (PRC), triethylene melamine (TEM), acrylamide monomer (AA), chlorambucil (CHL), melphalan (MLP), cyclophosphamide (CPP), diethyl sulfate (DES), ethyl methane sulfonate (EMS), methyl

methane sulfonate (MMS), 6-mercaptopurine (6MP), mitomycin-C (MMC), procarbazine (PRC), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), ^3H_2O, and urethane (UR)), and electromagnetic radiation (*e.g.*, X-ray radiation, gamma-radiation, ultraviolet light).

In one embodiment, equivalent cells within the scope of the invention include cells that are established from the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cells, and that have substantially the same sensitivity, increased sensitivity, or reduced sensitivity to SARS-CoV as the cell from which it is established. The term "sensitivity" and "sensitive" when made in reference to a cell is a relative term, which refers to the degree of permissiveness of the cell to a virus as compared to the degree of permissiveness of another cell to the same virus. For example, the term "increased sensitivity " to SARS-CoV when used in reference to the sensitivity of a first cell relative to a second cell refers to an increase in the first cell, preferably at least a 5%, more preferably from 5% to 10,000%, more preferably from 5% to 1,000%, yet more preferably from 10% to 200%, and even more preferably from 10% to 100%, increase in the quantity of SARS-CoV protein, SARS-CoV nucleic acid, and/or of CPE by progeny virus which is produced following infection of the first cell with SARS-CoV, as compared with the quantity of SARS-CoV protein, SARS-CoV nucleic acid, and/or of CPE by progeny virus (respectively) which is produced following infection of the second cell. For example, if 34 samples containing SARS-CoV were tested for the presence of progeny virus, with 25 and 13 samples showing the presence of CPE using a first cell and second cell, respectively, then the sensitivity is 74% and 38% for the first cell and second cell, respectively. This reflects an increase of 90% in the sensitivity of the first cell as compared to the sensitivity of the second cell.

In another embodiment, equivalent cells within the scope of the invention include cells that are established from the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cells, and that have substantially the same sensitivity to SARS-CoV as the cell from which it is established. This may be advantageous where, for example, the parent cell is made transgenic for a reporter gene.

In a further embodiment, equivalent cells within the scope of the invention include cells that are established from the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cells, and that have increased sensitivity or decreased sensitivity to SARS-CoV as compared to HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cells from which they were established. This may be desirable where, for example, the parent cell is made transgenic for a receptor gene, which alters the level of binding of SARS-CoV to the cell.

The invention's cells (such as the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) show the surprising property of being susceptible to, and permissive for, infection by SARS-CoV. The term "susceptible" as used herein in reference to a cell describes the ability of a permissive or non-permissive host cell to be infected by a virus. "Infection" refers to adsorption of the virus to the cell and penetration into the cell. A cell may be susceptible without being permissive, in that a cell may be penetrated by a virus in the absence of viral replication and/or release of virions from the cell. A permissive cell line however must be susceptible. Susceptibility of a cell to a virus may be determined by methods known in the art such as detecting the presence of viral proteins using electrophoretic analysis (*i.e.*, SDS-PAGE) of protein extracts prepared from the infected cell cultures. Susceptibility to SARS-CoV may also be determined by detecting the presence of SARS-CoV gRNA using the exemplary methods disclosed herein.

The terms "permissive" and "permissiveness" as used herein describe the sequence of interactive events between a virus and its putative host cell. The process begins with viral adsorption to the host cell surface and ends with release of infectious virions. A cell is "permissive" (*i.e.*, shows "permissiveness") if it is capable of supporting viral replication as determined by, for example, production of viral nucleic acid sequences and/or of viral peptide sequences, regardless of whether the viral nucleic acid sequences and viral peptide sequences are assembled into a virion. While not required, in one embodiment, a cell is permissive if it generates virions and/or releases the virions contained therein. Many methods are available for the determination of the permissiveness of a given cell line. For example, the replication of a particular virus in a host cell line may be measured by the production of various viral markers including viral proteins, viral nucleic acid (including both RNA and DNA) and the progeny virus. The presence of viral proteins may be determined using electrophoretic analysis (*i.e.*, SDS-PAGE) of protein extracts prepared from the infected cell cultures. Viral nucleic acid may be quantitated using nucleic acid hybridization assays. In a preferred embodiment, permissivity to SARS-CoV may also be determined by detecting the presence of SARS-CoV sgRNA using the exemplary methods disclosed herein. Susceptibility to SARS-CoV may also be determined by detecting the presence of SARS-CoV gRNA using the exemplary methods disclosed herein. Production of progeny virus may also be determined by observation of a cytopathic effect. However, this method is less preferred than detection of SARS-CoV sgRNA, since data herein shows that a cytopathic effect may not be observed even when viral replication is detectable by

sgRNA (Table 1). The invention is not limited to the specific quantity of replication of virus.

The terms "not permissive" and "non-infections" encompasses, for example, a cell that is not capable of supporting viral replication as determined by, for example, production of viral nucleic acid sequences and/or of viral peptide sequences, and/or assembly of viral nucleic acid sequences and viral peptide sequences into a virion.

The terms "cytopathic effect" and "CPE" as used herein describe changes in cellular structure (*i.e.*, a pathologic effect). Common cytopathic effects include cell destruction, syncytia (*i.e.*, fused giant cells) formation, cell rounding, vacuole formation, and formation of inclusion bodies. CPE results from actions of a virus on permissive cells that negatively affect the ability of the permissive cellular host to perform its required functions to remain viable. In *in vitro* cell culture systems, CPE is evident when cells, as part of a confluent monolayer, show regions of non-confluence after contact with a specimen that contains a virus. The observed microscopic effect is generally focal in nature and the foci are initiated by a single virion. However, depending upon viral load in the sample, CPE may be observed throughout the monolayer after a sufficient period of incubation. Cells demonstrating viral induced CPE usually change morphology to a rounded shape, and over a prolonged period of time can die and be released from their anchorage points in the monolayer. When many cells reach the point of focal destruction, the area is called a viral plaque, which appears as a hole in the monolayer. The terms "plaque" and "focus of viral infection" refer to a defined area of CPE which is usually the result of infection of the cell monolayer with a single infectious virus which then replicates and spreads to adjacent cells of the monolayer. Cytopathic effects are readily discernable and distinguishable by those skilled in the art.

In another embodiment, the invention contemplates the use of transgenic cells such as transgenic HEK-293T, transgenic Huh-7, transgenic Mv1Lu, transgenic pRHMK and/or transgenic pCMK cells. The terms "transgenic" and "genetically engineered" when made in reference to a cell, refer to a cell that has been transformed to contain a transgene. The term "transformation" as used herein refers to the introduction of a transgene into a cell by way of human intervention, using standard methods in the art. For example, where the nucleic acid sequence is a plasmid or naked piece of linear DNA, the sequence may be "transfected" into the cell using, for example, calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection,

liposome fusion, lipofection, protoplast fusion, and biolistics. Alternatively, where the nucleic acid sequence is encapsidated into a viral particle, the sequence may be introduced into a cell by "infecting" the cell with the virus.

Transformation of a cell may be stable or transient. The terms "transient transformation" and "transiently transformed" refer to the introduction of one or more nucleotide sequences of interest into a cell in the absence of integration of the nucleotide sequence of interest into the host cell's genome. Transient transformation may be detected by, for example, enzyme-linked immunosorbent assay (ELISA), which detects the presence of a polypeptide encoded by one or more of the nucleotide sequences of interest. Alternatively, transient transformation may be detected by detecting the activity of the protein (*e.g.*, β-glucuronidase) encoded by the nucleotide sequence of interest. The term "transient transformant" refer to a cell, which has transiently incorporated one or more nucleotide sequences of interest. Transient transformation with the invention's vectors may be desirable in, for example, cell biology or cell cycle investigations, which require efficient gene transfer.

In contrast, the terms "stable transformation" and "stably transformed" refer to the introduction and integration of one or more nucleotide sequence of interest into the genome of a cell. Thus, a "stable transformant" is distinguished from a transient transformant in that, whereas genomic DNA from the stable transformant contains one or more nucleotide sequences of interest, genomic DNA from the transient transformant does not contain the nucleotide sequence of interest. Stable transformation of a cell may be detected by Southern blot hybridization of genomic DNA of the cell with nucleic acid sequences, which are capable of binding to one or more of the nucleotide sequences of interest. Alternatively, stable transformation of a cell may also be detected by the polymerase chain reaction of genomic DNA of the cell to amplify the nucleotide sequence of interest.

A transgene that is introduced into the cells of the invention may comprise nucleotide sequence that is "endogenous" or "heterologous" (*i.e.*, "foreign"). The term "endogenous" refers to a sequence, which is naturally found in the cell or virus into which it is introduced so long as it does not contain some modification relative to the naturally-occurring sequence. The term "heterologous" refers to a sequence, which is not endogenous to the cell or virus into which it is introduced. For example, heterologous DNA includes a nucleotide sequence, which is ligated to, or is manipulated for ligation to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location

in nature. Heterologous DNA also includes a nucleotide sequence, which is naturally found in the cell or virus into which it is introduced and which contains some modification relative to the naturally-occurring sequence. Generally, although not necessarily, heterologous DNA encodes heterologous RNA and heterologous proteins that are not normally produced by the cell or virus into which it is introduced. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, DNA sequences which encode selectable marker proteins (*e.g.*, proteins which confer drug resistance), *etc.*

The term "wild-type" when made in reference to a peptide sequence and nucleotide sequence refers to a peptide sequence and nucleotide sequence, respectively, which has the characteristics of that peptide sequence and nucleotide sequence when isolated from a naturally occurring source. A wild-type peptide sequence and nucleotide sequence is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the peptide sequence and nucleotide sequence, respectively. In contrast, the term "modified" or "mutant" refers to a peptide sequence and nucleotide sequence which displays modifications in sequence and/or functional properties (*i.e.*, altered characteristics) when compared to the wild-type peptide sequence and nucleotide sequence, respectively. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type peptide sequence and nucleotide sequence. Nucleic acid sequences and/or proteins may be modified by chemical, biochemical, and/or molecular biological techniques. Modifications to nucleic acid sequences include introduction of one or more deletion, insertion, and substitution. A "deletion" is defined as a change in a nucleic acid sequence in which one or more nucleotides is absent. An "insertion" or "addition" is that change in a nucleic acid sequence, which has resulted in the addition of one or more nucleotides. A "substitution" results from the replacement of one or more nucleotides by one or more different nucleotides.

While not required, in one embodiment, it may be desirable that the transgene contains a sequence encoding a selectable marker. The term "selectable marker" as used herein refers to nucleotide sequence, which encodes an enzymatic activity that confers resistance to a compound (*e.g.*, antibiotic or drug) upon the cell in which the selectable marker is expressed. Selectable markers may be "positive"; i.e., genes, which encode an enzymatic activity that can be detected in any cell or cell line. Examples of dominant selectable markers include, but are not limited to, (1) the bacterial aminoglycoside 3'

phosphotransferase gene (also referred to as the neo gene) which confers resistance to the drug G418 in cells, (2) the bacterial hygromycin G phosphotransferase (hyg) gene which confers resistance to the antibiotic hygromycin, and (3) the bacterial xanthine-guanine phosphoribosyl transferase gene (also referred to as the gpt gene) which confers the ability to grow in the presence of mycophenolic acid. Other selectable markers are not dominant in that their use must be in conjunction with a cell line that lacks the relevant enzyme activity. Selectable markers may be "negative"; negative selectable markers encode an enzymatic activity whose expression is cytotoxic to the cell when grown in an appropriate selective medium. For example, the HSV-tk gene and the dt gene are commonly used as a negative selectable marker. Expression of the HSV-tk gene in cells grown in the presence of gancyclovir or acyclovir is cytotoxic; thus, growth of cells in selective medium containing gancyclovir or acyclovir selects against cells capable of expressing a functional HSV TK enzyme. Similarly, the expression of the dt gene selects against cells capable of expressing the Diphtheria toxin. In one embodiment, the selectable marker gene used is the neo gene in plasmid pcDNA3 (Invitrogen) and cells that incorporate this transgene may be selected by exposure to Geneticin (G418) (Gibco-BRL Inc.).

In another embodiment, it may be desirable that the transgene contains a sequence (*e.g.*, the *uid A* gene) encoding a reporter protein. This may be desirable where, for example, the reporter protein is more readily detectable than another protein to which it is operably linked. The term "reporter gene" refers to a gene, which encodes a reporter molecule (e.g., RNA, polypeptide, etc.), which is detectable in any detection system, including, but not limited to enzyme (e.g., ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. Exemplary reporter genes include, for example, β-glucuronidase gene, green fluorescent protein gene, *E. coli* β-galactosidase (LacZ) gene, *Halobacterium* β-galactosidase gene, *E. coli* luciferase gene, *Neuropsora* tyrosinase gene, Aequorin (jellyfish bioluminescence) gene, human placental alkaline phosphatase gene, and chloramphenicol acetyltransferase (CAT) gene. Reporter genes are commercially available, such as from Clontech, Invitrogen, and Promega. It is not intended that the present invention be limited to any particular detection system or label.

In a further embodiment, it may be desirable that the transgenic cell (such as transgenic cells of the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) expresses a probe gene. The term "probe" gene refers to a sequence useful in the detection, identification and/or isolation of particular polypeptide sequence. Exemplary

probe genes encode ligand-binding systems useful for the isolation of polypeptides such as the staphylococcal protein A and its derivative ZZ (which binds to human polyclonal IgG), histidine tails (which bind to Ni^{2+}), biotin (which binds to streptavidin), maltose-binding protein (MBP) (which binds to amylose), glutathione S-transferase (which binds to glutathione), *etc.* Exemplary probe gene sequences include reporter genes, as discussed above.

In yet another embodiment, the transgenic cell (such as the transgenic cell of an exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) may express a "fusion protein." Exemplary sequences that may be included in a fusion gene include those for adenosine deaminase (ADA) gene (GenBank Accession No. M13792); alpha-1-antitrypsin gene (GenBank Accession No. M11465); beta chain of hemoglobin gene (GenBank Accession No. NM_000518); receptor for low density lipoprotein gene (GenBank Accession No. D16494); lysosomal glucocerebrosidase gene (GenBank Accession No. K02920); hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene (GenBank Accession No. M26434, J00205, M27558, M27559, M27560, M27561, M29753, M29754, M29755, M29756, M29757); lysosomal arylsulfatase A (ARSA) gene (GenBank Accession No. NM_000487); ornithine transcarbamylase (OTC) gene (GenBank Accession No. NM_000531); phenylalanine hydroxylase (PAH) gene (GenBank Accession No. NM_000277); purine nucleoside phosphorylase (NP) gene (GenBank Accession No. NM_000270); the dystrophin gene (GenBank Accession Nos. M18533, M17154, and M18026); the utrophin (also called the dystrophin related protein) gene (GenBank Accession No. NM_007124); and the human cystic fibrosis transmembrane conductance regulator (CFTR) gene (GenBank Accession No.M28668).

In another embodiment, the transgenic cell (such as the transgenic cell of the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) may be transfected with nucleotide sequences encoding a cytokine. "Cytokine" refers to a molecule, such a protein or glycoprotein, involved in the regulation of cellular proliferation and function. Cytokines are exemplified by lymphokines (*e.g.*, tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), tumor necrosis factor-γ (TNF-γ), *etc.*), interferons such as interferon-γ (IFN-γ), tumor necrosis factor (TNF), *etc.*), growth factors (*e.g.*, erythropoietin, G-CSF, M-CSF, GM-CSF, epidermal growth factor (EGF), insulin, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), *etc.*), and interleukins (*e.g.*, interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5),

interleukin-6 (IL-6), interleukin-9 (IL-9), interleukin-10 (IL-10), and interleukin-13 (IL-13)).

The transgenic cells may be useful where it is desirable to determine the effect of the transgenic polypeptide on the cell's susceptibility and/or permissivity to SARS-CoV. For example, increased permissivity of the transgenic cell compared to the cell into which the transgene was introduced may be useful in generating higher virus titers and/or higher viral proteins for use in vaccine production and/or generation of antibodies. Conversely, reduced permissivity of the transgenic cell compared to the cell into which the transgene was introduced may be useful in reducing the risk of infection with SARS-CoV. For example, Mv1Lu cells are routinely used in diagnostic assays for the detection of influenza and/or parainfluenza viruses. Thus, a transgenic Mv1Lu cell with reduced permissivity to SARS-CoV compared to a Mv1Lu cell into which the transgene was introduced is safer to use in small laboratories for detection of influenza and/or parainfluenza viruses without the need to resort to containment approaches that would otherwise be required for cells producing infectious SARS-CoV. Thus, in one embodiment, the Mv1Lu cells (whether or not they are transgenic) retain their susceptibility to one or more of influenza virus and parainfluenza virus.

In a further embodiment, the transgenic cell (such as the transgenic cell of the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) may be engineered to include other nucleotide sequences of interest, such as non-coding regulatory sequences, which do not encode an mRNA or protein product, (*e.g.*, promoter sequence, polyadenylation sequence, termination sequence, enhancer sequence, *etc.*). Exemplary "promoters" include, without limitation, single, double and triple promoters.

In a further embodiment, the transgenic cell (such as the transgenic cell of the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) expresses a receptor gene. The term "receptor" refers to a structure (generally, but not necessarily, a protein) located on or in a cell, which specifically recognizes a binding molecule (i.e., a ligand). In one embodiment, this binding initiates either a specific biological response or the transduction of a signal. However, it is not necessary that binding result in a specific biological response or the transduction of a signal, as for example when a virus binds to a receptor on a cell.

In one embodiment, the transgenic cell is a Mv1Lu-hF cell (ATCC accession No. PTA-4737), *i.e.*, a transgenic Mv1Lu that expresses human furin, as described in U.S. Patent No. 6,610,474, which is incorporated by reference in its entirety.

In a further embodiment, the transgenic cell comprises a cell line established from a transgenic cell line designated Mv1Lu-hF, wherein the established cell line has a property selected from the group consisting of (a) increased sensitivity to at least one virus selected from the group consisting of influenza A virus, influenza B virus and parainfluenza virus 3, as compared to the Mv1Lu cell line, and (b) enhanced productivity of infectious virions upon inoculation with at least one virus selected from the group one consisting of influenza A virus, influenza B virus and parainfluenza virus 3, as compared to the Mv1Lu cell line. In a more preferred embodiment, the cell line has the sensitivity of the cell line designated Mv1Lu-hF, to at least one virus selected from the group consisting of influenza A virus, influenza B virus and parainfluenza virus 3. In an alternative embodiment, the transgenic cell comprises a transgenic mink lung epithelial cell line expressing human furin, wherein the cell line has a property selected from the group consisting of (a) increased sensitivity to at least one virus selected from the group consisting of influenza A virus, influenza B virus and parainfluenza virus 3, as compared to Mv1Lu, and (b) enhanced productivity of infectious virions upon inoculation with at least one virus selected from the group one consisting of influenza A virus, influenza B virus and parainfluenza virus 3, as compared to Mv1Lu. More preferably, transgenic mink lung epithelial cell line has the sensitivity of the cell line designated Mv1Lu-hF and deposited as ATCC accession number PTA-4737, to at least one virus selected from the group consisting of influenza A virus, influenza B virus and parainfluenza virus 3. Each of these transgenic cells is described in U.S. Patent No. 6,610,474, which is incorporated by reference in its entirety

F. Exemplary Assays For Detecting Replication Of SARS-CoV

The invention provides a method for detecting replication of SARS-coronavirus in a sample, comprising detecting the presence SARS-coronavirus sgRNA in the sample. These methods are useful in, for example, diagnosing the presence of SARS-CoV, identifying cells that are susceptible and/or permissive for SARS-CoV, screening agents that alter infection with SARS-CoV, and in determining the relative efficacy of agents and/or modalities of treatment in altering SARS infection.

One aspect that distinguishes the invention's methods from the prior art is that the invention's methods detect the presence of sgRNA, whereas prior art methods relied on detection of gRNA, which only detected virus input, but could not distinguish this from viral RNA replication and mRNA production (Drosten, et al., 2003, N.Engl.J.Med. 348:1967-1976; Poon, et al., 2003, Clin.Chem. 49:953-955; Poutanen, et al., 2003, N.Engl.J.Med. 348:1995-2005). Thus, the invention's assay can differentiate non-replicating genomic SARS-CoV RNA from the replicative forms produced during an active infection. The assay could therefore be used to differentiate exposure (or mechanical transmission in an animal vector) from active infection and or viral replication.

Also, the invention's methods that utilize detection of sgRNA are distinguished from prior methods that use CPE for detection of virus in that data herein (Table 1) confirms that CPE is not an accurate indicator of SARS-CoV replication, whereas detection of sgRNA reproducibly detected such replication. The invention's use of sgRNA to identify an active SARS-CoV infection is not currently used to diagnose human and/or animal coronaviruses of veterinary importance. The invention's methods are also distinguished from methods using RACE assay (Zeng et al. (2003) Exp. Biol. Med. 228(7):866-73).

The detection of sgRNA in accordance with the invention's methods are suitable for detection of early replication of SARS-CoV. One utility and advantage of this method is that, when coupled with titration of viral supernatants, cells permissive to SARS-CoV can be identified. As disclosed herein (Examples 3, 5 and 6), the invention's methods have successfully identified monkey, mink and human cells that are susceptible and permissive to SARS-CoV infection. The finding that SARS-CoV enters various cell types and initiates replication is useful as the basis for the development of diagnostic assays, especially when coupled with a SARS-CoV-specific nucleic acid and/or SARS-CoV-specific antigen detection methods. Where RNA replication results in the production of infectious virions, the permissive cell lines are also useful candidates for vaccine production. Identification of cell lines that result in abortive replication will lead to more sensitive and/or safer diagnostic cells that can be used as antigen sources and for identifying potential anti-SARS-CoV drug targets.

Additionally, cells that are susceptible to SARS-CoV binding and entry, but that have blocks between the initiation of replication and the production of new virus, can also be identified using the invention's methods. The use of molecular diagnostic methods such as nucleic acid probes and monoclonal antibodies has however, demonstrated that non-

permissive cells may have the ability to provide a cell-based test for detecting the presence of a virus. In fact, when considering detection of viruses that require level III biological containment for cell culture amplification, *e.g.* SARS coronavirus, a cell line that does not produce and release infectious virus to a high level may have substantial advantages in safety. An example from the art of the diagnostic use of a non-permissive cell line is the use of mink lung cells (Mv1Lu) to detect cytomegalovirus (CMV) (Gleaves, et al., J Clin Microbiol, 1992. 30(4): p. 1045-8). Human embryonic lung cells (MRC-5) are considered the cell line of choice to produce infectious CMV virus, however mink lung cells have been shown to be useful in detecting the primary infection event of CMV by using a monoclonal antibody that targets the CMV immediate early (mIE Ag) protein that is produced in abundant amounts. Reported benefits of the non-permissive Mv1Lu cells over MRC-5 cells were higher detection sensitivity and lower toxicity from non-specific material present in the clinical specimen. Mink lung cells have also been shown to be commercially useful for influenza virus detection.

i. sgRNA

In one embodiment, the invention's methods detect sgRNA. The terms "subgenomic RNA" and "sgRNA" are used interchangeably herein to refer to a partial genomic sequence (*e.g.*, coronavirus individual messenger RNA sequences) comprising at least a portion of a leader sequence.

The term "leader sequence" refers to a sequence of about 40 to about 150, about 50 to about 80, and or about 55 to about 75 nucleotides that is located at the 5' terminus of the genome. This sequence is juxtaposed to the 5' terminus of each subgenomic RNA by transcriptional mechanisms during synthesis. There is very strong sequence conservation of the leader sequence across the strains of SARS (Drosten et al., N.Engl.J.Med. 2003;1967-76; Ksiazek et al., N.Engl.J.Med. 2003;1953-66; Marra et al., Science 2003;1399-404). The leader sequence plays a role in the generation of the subgenomic RNA transcripts (Holmes et al., In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, eds. Fields Virology. Philadelphia: Lippincott Williams & Wilkins, 2001;1187-1203; Holmes et al. "Coronaviridae: The viruses and their replication." In: Fields BN, Knipe DM, Howely PM, eds. Fields Virology. Philadelphia: Lippincott-Raven, 1996;1075-1094; Sawicki et al., J.Gen.Virol. 2001;385-96; Sawicki et al.,

Adv.Exp.Med.Biol. 1998;215-9; Wang et al., Adv.Exp.Med.Biol. 2001;491-7), transcription, replication, translation and/or packaging of viral RNA.

Sequence alignment by the inventors showed conservation of at least a portion of the leader sequence in the following exemplary strains of SARS-CoV: gi|31416292|gb|AY278487.3| SARS coronavirus BJ02, gi|30248028|gb|AY274119.3| SARS coronavirus TOR2, gi|30698326|gb|AY291451.1| SARS coronavirus TW1, gi|33115118|gb|AY323977.2| SARS coronavirus HSR 1, gi|35396382|gb|AY394850.1| SARS coronavirus WHU, gi|33411459|dbj|AP006561.1| SARS coronavirus TWY, gi|33411444|dbj|AP006560.1| SARS coronavirus TWS, gi|33411429|dbj|AP006559.1| SARS coronavirus TWK, gi|33411414|dbj|AP006558.1| SARS coronavirus TWJ, gi|33411399|dbj|AP006557.1| SARS coronavirus TWH, gi|30023963|gb|AY278491.2| SARS coronavirus HKU-39849, gi|33578015|gb|AY310120.1| SARS coronavirus FRA, gi|33518725|gb|AY362699.1| SARS coronavirus TWC3, gi|33518724|gb|AY362698.1| SARS coronavirus TWC2, gi|30027617|gb|AY278741.1| SARS coronavirus Urbani, gi|31873092|gb|AY321118.1| SARS coronavirus TWC, gi|33304219|gb|AY351680.1| SARS coronavirus ZMY 1, gi|31416305|gb|AY278490.3| SARS coronavirus BJ03, gi|30910859|gb|AY297028.1| SARS coronavirus ZJ01, gi|30421451|gb|AY282752.1| SARS coronavirus CUHK-Su10, gi|34482146|gb|AY304495.1| SARS coronavirus GZ50, gi|34482139|gb|AY304488.1| SARS coronavirus SZ16, gi|34482137|gb|AY304486.1| SARS coronavirus SZ3, gi|30027610|gb|AY278554.2| SARS coronavirus CUHK-W1, gi|31416306|gb|AY279354.2| SARS coronavirus BJ04, gi|37576845|gb|AY427439.1| SARS coronavirus AS, gi|37361915|gb|AY283798.2| SARS coronavirus Sin2774, gi|31416290|gb|AY278489.2| SARS coronavirus GD01, gi|30468042|gb|AY283794.1| SARS coronavirus Sin2500, gi|30468043|gb|AY283795.1| SARS coronavirus Sin2677, gi|30468044|gb|AY283796.1| SARS coronavirus Sin2679, gi|30468045|gb|AY283797.1| SARS coronavirus Sin2748, gi|31982987|gb|AY286320.2| SARS coronavirus isolate ZJ-HZ01, gi|30275666|gb|AY278488.2| SARS coronavirus BJ01.

In one embodiment, the leader sequence is exemplified by the sequence from nucleotide 1 to nucleotide 72 for SARS-CoV (Urbani) (Figure 7): 5'-atattaggtttttac ctacccaggaaaagccaaccaacctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:36); 5'-tattaggtttttacctacccaggaaaagccaaccaacctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:37) of gi|33304219|gb|AY351680.1| SARS coronavirus ZMY 1, 5'-taggtttttacctacccaggaaaagccaaccaacctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:38) of

gi|31416305|gb|AY278490.3| SARS coronavirus BJ03, 5'-ctacccaggaaaagccaaccaacctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:77) of gi|30421451|gb|AY282752.1| SARS coronavirus CUHK-Su10, 5'-tacccaggaaaagccaaccaacctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:78) of gi|31416306|gb|AY279354.2| SARS coronavirus BJ04, and 5'-ccaggaaaagccaaccaacctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:79) of gi|30275666|gb|AY278488.2| SARS coronavirus BJ01.

As disclosed herein, portions of the leader sequence are expressly contemplated as equivalents to the full length leader sequence for the detection of sgRNA and/or SARS-CoV replication. Exemplary portions of the SARS-CoV (Urbani) leader sequence include, without limitation, 5'-atattaggtttttacctacccaggaaaagccaaccaacctcgatctcttgtagatctgttct-3' (SEQ ID NO:39), 5'-atattaggtttttacctacccaggaaaagccaaccaacctcgatctcttgtagatct-3' (SEQ ID NO:40), 5'-atattaggtttttacctacccaggaaaagccaaccaacctcgatctcttgtag-3' (SEQ ID NO:41), 5'-atattaggtttttacctacccaggaaaagccaaccaacctcgatc-3' (SEQ ID NO:42), 5'-atattaggtttttacctacccaggaaaagccaaccaacc-3' (SEQ ID NO:43), 5'-atattaggtttttacctacccaggaaaagccaac-3' (SEQ ID NO:44), 5'-atattaggtttttacctacccaggaaaagc-3' (SEQ ID NO:45), 5'-atattaggtttttacctacccagg-3' (SEQ ID NO:46), 5'-atattaggtttttacctac-3' (SEQ ID NO:47), 5'-atattagg-3' (SEQ ID NO:48), 5'-ttacctacccaggaaaagccaaccaacctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:49), 5'-aaaagccaaccaacctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:50), 5'-gccaaccaacctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:51), 5'-ccaacctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:52), 5'-ctcgatctcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:53), 5'-tcttgtagatctgttctctaaacgaac-3' (SEQ ID NO:54), 5'-gatctgttctctaaacgaac-3' (SEQ ID NO:55), and 5'-taaacgaac-3' (SEQ ID NO:56), 5'- atattaggtt tttacctacc caggaaaagc caaccaacct cgatctcttg tagatctgtt -3' (SEQ ID NO:57), 5'- atattaggtt tttacctacc caggaaaagc caaccaacct cgatctcttg -3' (SEQ ID NO:58), 5'-atattaggtt tttacctacc caggaaaagc caaccaacct -3' (SEQ ID NO:59), 5'- atattaggtt tttacctacc caggaaaagc -3' (SEQ ID NO:60), 5'- atattaggtt tttacctacc-3' (SEQ ID NO:61), 5'- atattaggtt -3' (SEQ ID NO:62), 5'- tttacctacc caggaaaagc caaccaacct cgatctcttg tagatctgtt ctctaaacga ac-3' (SEQ ID NO:63), 5'- caggaaaagc caaccaacct cgatctcttg tagatctgtt ctctaaacga ac-3' (SEQ ID NO:64), 5'- caaccaacct cgatctcttg tagatctgtt ctctaaacga ac-3' (SEQ ID NO:65), 5'-cgatctcttg tagatctgtt ctctaaacga ac-3' (SEQ ID NO:66), 5'- tagatctgtt ctctaaacga ac-3' (SEQ ID NO:67), and 5'- ctctaaacga ac-3' (SEQ ID NO:68).

In one embodiment, the sgRNA comprises at least a portion of a leader sequence operably linked to at least a portion of a gene encoding a SARS-CoV polypeptide. The term "polypeptide," "protein," "peptide," "peptide sequence," "amino acid sequence," and "polypeptide sequence" are used interchangeably herein to refer to at least two amino acids or amino acid analogs which are covalently linked by a peptide bond or an analog of a peptide bond. The term peptide includes oligomers and polymers of amino acids or amino acid analogs. The term peptide also includes molecules, which are commonly referred to as peptides, which generally contain from about two (2) to about twenty (20) amino acids. The term peptide also includes molecules, which are commonly referred to as polypeptides, which generally contain from about twenty (20) to about fifty amino acids (50). The term peptide also includes molecules, which are commonly referred to as proteins, which generally contain from about fifty (50) to about three thousand (3000) amino acids. The amino acids of the peptide may be L-amino acids or D-amino acids. A peptide, polypeptide or protein may be synthetic, recombinant or naturally occurring. A synthetic peptide is a peptide, which is produced by artificial means *in vitro* (*e.g.*, was not produced *in vivo*).

The term "SARS-CoV polypeptide" refers to any polypeptide that is encoded by the SARS-CoV genome (regardless of whether the genome is "wild type" or "modified"), including, for example, antigenic polypeptides. SARS-CoV polypeptides are exemplified by, but not limited to, Nucleocapsid (N), Spike glycoprotein (S), Matrix (M), E protein, and Replicase proteins (Pol 1a/b).

The "Nucleocapsid" protein (also referred to as "N") refers to a protein that is produced early in infection and at very high abundance. The N of other CoVs is highly immunogenic, eliciting antibodies and T-cell responses in natural infections. The Nucleocapsid protein is exemplified, but not limited to, the sequences in Figures 23-26 and 40, and those encoded by the genomic sequences in gi|31416292|gb|AY278487.3| SARS coronavirus BJ02, gi|30248028|gb|AY274119.3| SARS coronavirus TOR2, gi|30698326|gb|AY291451.1| SARS coronavirus TW1, gi|33115118|gb|AY323977.2| SARS coronavirus HSR 1, gi|35396382|gb|AY394850.1| SARS coronavirus WHU, gi|33411459|dbj|AP006561.1| SARS coronavirus TWY, gi|33411444|dbj|AP006560.1| SARS coronavirus TWS, gi|33411429|dbj|AP006559.1| SARS coronavirus TWK, gi|33411414|dbj|AP006558.1| SARS coronavirus TWJ, gi|33411399|dbj|AP006557.1| SARS coronavirus TWH, gi|30023963|gb|AY278491.2| SARS coronavirus HKU-39849, gi|33578015|gb|AY310120.1| SARS coronavirus FRA, gi|33518725|gb|AY362699.1| SARS

coronavirus TWC3, gi|33518724|gb|AY362698.1| SARS coronavirus TWC2, gi|30027617|gb|AY278741.1| SARS coronavirus Urbani, gi|31873092|gb|AY321118.1| SARS coronavirus TWC, gi|33304219|gb|AY351680.1| SARS coronavirus ZMY 1, gi|31416305|gb|AY278490.3| SARS coronavirus BJ03, gi|30910859|gb|AY297028.1| SARS coronavirus ZJ01, gi|30421451|gb|AY282752.1| SARS coronavirus CUHK-Su10, gi|34482146|gb|AY304495.1| SARS coronavirus GZ50, gi|34482139|gb|AY304488.1| SARS coronavirus SZ16, gi|34482137|gb|AY304486.1| SARS coronavirus SZ3, gi|30027610|gb|AY278554.2| SARS coronavirus CUHK-W1, gi|31416306|gb|AY279354.2| SARS coronavirus BJ04, gi|37576845|gb|AY427439.1| SARS coronavirus AS, gi|37361915|gb|AY283798.2| SARS coronavirus Sin2774, gi|31416290|gb|AY278489.2| SARS coronavirus GD01, gi|30468042|gb|AY283794.1| SARS coronavirus Sin2500, gi|30468043|gb|AY283795.1| SARS coronavirus Sin2677, gi|30468044|gb|AY283796.1| SARS coronavirus Sin2679, gi|30468045|gb|AY283797.1| SARS coronavirus Sin2748, gi|31982987|gb|AY286320.2| SARS coronavirus isolate ZJ-HZ01, and gi|30275666|gb|AY278488.2| SARS coronavirus BJ01.

The "Spike glycoprotein" (also referred to as "S") refers to a viral attachment protein that protrudes from the virion and that is a major antigenic determinant. Antibodies to this protein may neutralize the virus rendering it non infectious. The Spike glycoprotein is exemplified, but not limited to, the sequences in Figures 27-29 and 41, and by those encoded by the genomic sequences in gi|31416292|gb|AY278487.3| SARS coronavirus BJ02, gi|30248028|gb|AY274119.3| SARS coronavirus TOR2, gi|30698326|gb|AY291451.1| SARS coronavirus TW1, gi|33115118|gb|AY323977.2| SARS coronavirus HSR 1, gi|35396382|gb|AY394850.1| SARS coronavirus WHU, gi|33411459|dbj|AP006561.1| SARS coronavirus TWY, gi|33411444|dbj|AP006560.1| SARS coronavirus TWS, gi|33411429|dbj|AP006559.1| SARS coronavirus TWK, gi|33411414|dbj|AP006558.1| SARS coronavirus TWJ, gi|33411399|dbj|AP006557.1| SARS coronavirus TWH, gi|30023963|gb|AY278491.2| SARS coronavirus HKU-39849, gi|33578015|gb|AY310120.1| SARS coronavirus FRA, gi|33518725|gb|AY362699.1| SARS coronavirus TWC3, gi|33518724|gb|AY362698.1| SARS coronavirus TWC2, gi|30027617|gb|AY278741.1| SARS coronavirus Urbani, gi|31873092|gb|AY321118.1| SARS coronavirus TWC, gi|33304219|gb|AY351680.1| SARS coronavirus ZMY 1, gi|31416305|gb|AY278490.3| SARS coronavirus BJ03, gi|30910859|gb|AY297028.1| SARS coronavirus ZJ01, gi|30421451|gb|AY282752.1| SARS coronavirus CUHK-Su10,

gi|34482146|gb|AY304495.1| SARS coronavirus GZ50, gi|34482139|gb|AY304488.1| SARS coronavirus SZ16, gi|34482137|gb|AY304486.1| SARS coronavirus SZ3, gi|30027610|gb|AY278554.2| SARS coronavirus CUHK-W1, gi|31416306|gb|AY279354.2| SARS coronavirus BJ04, gi|37576845|gb|AY427439.1| SARS coronavirus AS, gi|37361915|gb|AY283798.2| SARS coronavirus Sin2774, gi|31416290|gb|AY278489.2| SARS coronavirus GD01, gi|30468042|gb|AY283794.1| SARS coronavirus Sin2500, gi|30468043|gb|AY283795.1| SARS coronavirus Sin2677, gi|30468044|gb|AY283796.1| SARS coronavirus Sin2679, gi|30468045|gb|AY283797.1| SARS coronavirus Sin2748, gi|31982987|gb|AY286320.2| SARS coronavirus isolate ZJ-HZ01, and gi|30275666|gb|AY278488.2| SARS coronavirus BJ01.

The terms "Matrix protein," "Membrane protein," "membrane glycoprotein," and "M protein" refer to a protein that makes the shell of the virus and is closely associated with the lipid membrane that is acquired from the host cell. This protein is also made in abundance in an infected cell and there is typically a strong immune response (particularly, antibody response) to M in CoV infections. The Matrix protein is exemplified, but not limited to, the sequences in Figure 30, and by those encoded by the genomic sequences in gi|31416292|gb|AY278487.3| SARS coronavirus BJ02, gi|30248028|gb|AY274119.3| SARS coronavirus TOR2, gi|30698326|gb|AY291451.1| SARS coronavirus TW1, gi|33115118|gb|AY323977.2| SARS coronavirus HSR 1, gi|35396382|gb|AY394850.1| SARS coronavirus WHU, gi|33411459|dbj|AP006561.1| SARS coronavirus TWY, gi|33411444|dbj|AP006560.1| SARS coronavirus TWS, gi|33411429|dbj|AP006559.1| SARS coronavirus TWK, gi|33411414|dbj|AP006558.1| SARS coronavirus TWJ, gi|33411399|dbj|AP006557.1| SARS coronavirus TWH, gi|30023963|gb|AY278491.2| SARS coronavirus HKU-39849, gi|33578015|gb|AY310120.1| SARS coronavirus FRA, gi|33518725|gb|AY362699.1| SARS coronavirus TWC3, gi|33518724|gb|AY362698.1| SARS coronavirus TWC2, gi|30027617|gb|AY278741.1| SARS coronavirus Urbani, gi|31873092|gb|AY321118.1| SARS coronavirus TWC, gi|33304219|gb|AY351680.1| SARS coronavirus ZMY 1, gi|31416305|gb|AY278490.3| SARS coronavirus BJ03, gi|30910859|gb|AY297028.1| SARS coronavirus ZJ01, gi|30421451|gb|AY282752.1| SARS coronavirus CUHK-Su10, gi|34482146|gb|AY304495.1| SARS coronavirus GZ50, gi|34482139|gb|AY304488.1| SARS coronavirus SZ16, gi|34482137|gb|AY304486.1| SARS coronavirus SZ3, gi|30027610|gb|AY278554.2| SARS coronavirus CUHK-W1, gi|31416306|gb|AY279354.2| SARS coronavirus BJ04, gi|37576845|gb|AY427439.1| SARS

coronavirus AS, gi|37361915|gb|AY283798.2| SARS coronavirus Sin2774, gi|31416290|gb|AY278489.2| SARS coronavirus GD01, gi|30468042|gb|AY283794.1| SARS coronavirus Sin2500, gi|30468043|gb|AY283795.1| SARS coronavirus Sin2677, gi|30468044|gb|AY283796.1| SARS coronavirus Sin2679, gi|30468045|gb|AY283797.1| SARS coronavirus Sin2748, gi|31982987|gb|AY286320.2| SARS coronavirus isolate ZJ-HZ01, and gi|30275666|gb|AY278488.2| SARS coronavirus BJ01.

The terms "E protein," "envelope protein," and "small envelope E protein" refer to a protein that interacts with the M protein, and that it is believed to aid in particle formation. It is present at low levels in the virus particle and at higher levels in an infected cell. The E protein is exemplified, but not limited to, the sequences in Figure 31, and by those encoded by the genomic sequences in gi|31416292|gb|AY278487.3| SARS coronavirus BJ02, gi|30248028|gb|AY274119.3| SARS coronavirus TOR2, gi|30698326|gb|AY291451.1| SARS coronavirus TW1, gi|33115118|gb|AY323977.2| SARS coronavirus HSR 1, gi|35396382|gb|AY394850.1| SARS coronavirus WHU, gi|33411459|dbj|AP006561.1| SARS coronavirus TWY, gi|33411444|dbj|AP006560.1| SARS coronavirus TWS, gi|33411429|dbj|AP006559.1| SARS coronavirus TWK, gi|33411414|dbj|AP006558.1| SARS coronavirus TWJ, gi|33411399|dbj|AP006557.1| SARS coronavirus TWH, gi|30023963|gb|AY278491.2| SARS coronavirus HKU-39849, gi|33578015|gb|AY310120.1| SARS coronavirus FRA, gi|33518725|gb|AY362699.1| SARS coronavirus TWC3, gi|33518724|gb|AY362698.1| SARS coronavirus TWC2, gi|30027617|gb|AY278741.1| SARS coronavirus Urbani, gi|31873092|gb|AY321118.1| SARS coronavirus TWC, gi|33304219|gb|AY351680.1| SARS coronavirus ZMY 1, gi|31416305|gb|AY278490.3| SARS coronavirus BJ03, gi|30910859|gb|AY297028.1| SARS coronavirus ZJ01, gi|30421451|gb|AY282752.1| SARS coronavirus CUHK-Su10, gi|34482146|gb|AY304495.1| SARS coronavirus GZ50, gi|34482139|gb|AY304488.1| SARS coronavirus SZ16, gi|34482137|gb|AY304486.1| SARS coronavirus SZ3, gi|30027610|gb|AY278554.2| SARS coronavirus CUHK-W1, gi|31416306|gb|AY279354.2| SARS coronavirus BJ04, gi|37576845|gb|AY427439.1| SARS coronavirus AS, gi|37361915|gb|AY283798.2| SARS coronavirus Sin2774, gi|31416290|gb|AY278489.2| SARS coronavirus GD01, gi|30468042|gb|AY283794.1| SARS coronavirus Sin2500, gi|30468043|gb|AY283795.1| SARS coronavirus Sin2677, gi|30468044|gb|AY283796.1| SARS coronavirus Sin2679, gi|30468045|gb|AY283797.1| SARS coronavirus Sin2748,

gi|31982987|gb|AY286320.2| SARS coronavirus isolate ZJ-HZ01, and gi|30275666|gb|AY278488.2| SARS coronavirus BJ01.

The terms "Replicase protein," "polyprotein 1a," "polypeptide 1a," "polypeptide 1b," "polyprotein 1b," "polyprotein 1ab" and "Pol 1a/b" refer to a relatively large polyprotein that is produced upon infection of a cell by coronaviruses, and that encodes proteins required for genome replication. The polyprotein is encoded by about the 5' two thirds of the genome and it is produced very early during a CoV infection. The polyprotein is autocatalytically cleaved by encoded proteases (*e.g.*, 3C-like protease) into many proteins that are present in an infected cell but are not packaged in the virus particle. These include, without limitation, the RNA dependent RNA polymerase, a helicase and proteases (e.g., 3C-like, Pl1 and Pl2). The polyprotein 1a, 1b and 1ab are exemplified, but not limited to, the sequences in Figures 32-39, and by those encoded by the genomic sequences in gi|31416292|gb|AY278487.3| SARS coronavirus BJ02, gi|30248028|gb|AY274119.3| SARS coronavirus TOR2, gi|30698326|gb|AY291451.1| SARS coronavirus TW1, gi|33115118|gb|AY323977.2| SARS coronavirus HSR 1, gi|35396382|gb|AY394850.1| SARS coronavirus WHU, gi|33411459|dbj|AP006561.1| SARS coronavirus TWY, gi|33411444|dbj|AP006560.1| SARS coronavirus TWS, gi|33411429|dbj|AP006559.1| SARS coronavirus TWK, gi|33411414|dbj|AP006558.1| SARS coronavirus TWJ, gi|33411399|dbj|AP006557.1| SARS coronavirus TWH, gi|30023963|gb|AY278491.2| SARS coronavirus HKU-39849, gi|33578015|gb|AY310120.1| SARS coronavirus FRA, gi|33518725|gb|AY362699.1| SARS coronavirus TWC3, gi|33518724|gb|AY362698.1| SARS coronavirus TWC2, gi|30027617|gb|AY278741.1| SARS coronavirus Urbani, gi|31873092|gb|AY321118.1| SARS coronavirus TWC, gi|33304219|gb|AY351680.1| SARS coronavirus ZMY 1, gi|31416305|gb|AY278490.3| SARS coronavirus BJ03, gi|30910859|gb|AY297028.1| SARS coronavirus ZJ01, gi|30421451|gb|AY282752.1| SARS coronavirus CUHK-Su10, gi|34482146|gb|AY304495.1| SARS coronavirus GZ50, gi|34482139|gb|AY304488.1| SARS coronavirus SZ16, gi|34482137|gb|AY304486.1| SARS coronavirus SZ3, gi|30027610|gb|AY278554.2| SARS coronavirus CUHK-W1, gi|31416306|gb|AY279354.2| SARS coronavirus BJ04, gi|37576845|gb|AY427439.1| SARS coronavirus AS, gi|37361915|gb|AY283798.2| SARS coronavirus Sin2774, gi|31416290|gb|AY278489.2| SARS coronavirus GD01, gi|30468042|gb|AY283794.1| SARS coronavirus Sin2500, gi|30468043|gb|AY283795.1| SARS coronavirus Sin2677, gi|30468044|gb|AY283796.1| SARS coronavirus Sin2679, gi|30468045|gb|AY283797.1|

SARS coronavirus Sin2748, gi|31982987|gb|AY286320.2| SARS coronavirus isolate ZJ-HZ01, and gi|30275666|gb|AY278488.2| SARS coronavirus BJ01.

As disclosed herein, in one preferred embodiment, the sgRNA comprises a leader sequence operably linked to the amino terminal region of the Spike protein. This sequence may be amplified by RT-PCR using the primers SARS-1 (5'-ATATTAGGTTTTTACCTACCCAGG-3') (SEQ ID NO:69) which binds to the leader sequence from nucleotides 1-24 and primer SARS-21,593R (5'-AGTATGTTGAGTGTAATTAGGAG-3') (SEQ ID NO:70) which binds to nucleotides encoding the Spike glycoprotein.

ii. gRNA

The invention's methods may further comprise detecting SARS-coronavirus gRNA. The terms "genomic RNA" and "gRNA" are used interchangeably to refer to at least a portion of the genomic sequence such as that exemplified by the genome sequences of SARS coronavirus Urbani (GenBank accession # AY278741, Figure 7), SARS coronavirus Tor2 (GenBank accession # AY274119, Figure 8), SARS coronavirus CUHK-W1 (GenBank accession # AY278554, Figure 9), SARS-CoV Shanhgai LY (GenBank accession # H012999, Figures 10-13; GenBank accession # AY322205, Figure 20; GenBank accession # AY322206, Figure 21), SARS-CoV Shanghai QXC (GenBank accession # AH013000, Figures 14-16; GenBank accession # AY322208, Figure 17; GenBank accession # AY322197, Figure 18; GenBank accession # AY322199, Figure 19), and SARS-CoV ZJ-HZ01 (GenBank accession # AY322206, Figure 22), gi|31416292|gb|AY278487.3| SARS coronavirus BJ02, gi|30248028|gb|AY274119.3| SARS coronavirus TOR2, gi|30698326|gb|AY291451.1| SARS coronavirus TW1, gi|33115118|gb|AY323977.2| SARS coronavirus HSR 1, gi|35396382|gb|AY394850.1| SARS coronavirus WHU, gi|33411459|dbj|AP006561.1| SARS coronavirus TWY, gi|33411444|dbj|AP006560.1| SARS coronavirus TWS, gi|33411429|dbj|AP006559.1| SARS coronavirus TWK, gi|33411414|dbj|AP006558.1| SARS coronavirus TWJ, gi|33411399|dbj|AP006557.1| SARS coronavirus TWH, gi|30023963|gb|AY278491.2| SARS coronavirus HKU-39849, gi|33578015|gb|AY310120.1| SARS coronavirus FRA, gi|33518725|gb|AY362699.1| SARS coronavirus TWC3, gi|33518724|gb|AY362698.1| SARS coronavirus TWC2, gi|30027617|gb|AY278741.1| SARS coronavirus Urbani, gi|31873092|gb|AY321118.1| SARS coronavirus TWC, gi|33304219|gb|AY351680.1| SARS

coronavirus ZMY 1, gi|31416305|gb|AY278490.3| SARS coronavirus BJ03, gi|30910859|gb|AY297028.1| SARS coronavirus ZJ01, gi|30421451|gb|AY282752.1| SARS coronavirus CUHK-Su10, gi|34482146|gb|AY304495.1| SARS coronavirus GZ50, gi|34482139|gb|AY304488.1| SARS coronavirus SZ16, gi|34482137|gb|AY304486.1| SARS coronavirus SZ3, gi|30027610|gb|AY278554.2| SARS coronavirus CUHK-W1, gi|31416306|gb|AY279354.2| SARS coronavirus BJ04, gi|37576845|gb|AY427439.1| SARS coronavirus AS, gi|37361915|gb|AY283798.2| SARS coronavirus Sin2774, gi|31416290|gb|AY278489.2| SARS coronavirus GD01, gi|30468042|gb|AY283794.1| SARS coronavirus Sin2500, gi|30468043|gb|AY283795.1| SARS coronavirus Sin2677, gi|30468044|gb|AY283796.1| SARS coronavirus Sin2679, gi|30468045|gb|AY283797.1| SARS coronavirus Sin2748, gi|31982987|gb|AY286320.2| SARS coronavirus isolate ZJ-HZ01, and gi|30275666|gb|AY278488.2| SARS coronavirus BJ01.

Exemplary genomic RNA includes, without limitation, at least a portion of orf1ab polyprotein, orf1a polyprotein, Spike glycoprotein, Orf3a, Orf3a, Orf4b, Orf6, Orf7a, Orf7b, Orf8A, Orf8b, Nucleocapsid protein, Envelope protein E, and Membrane glycoprotein M.

In one preferred embodiment, the gRNA is at least a portion of the Polyprotein 1ab (also referred to as Polypeptide 1ab) gene. In one embodiment, detection of at least a portion of this gene distinguishes between sgRNA and gRNA, while detection 3' to the polyprotein 1ab gene detects both gRNA and sgRNA, without distinguishing between them. In one embodiment, the gRNA is of the polyprotein 1ab gene nucleotides from about 1 to about 21,485 of the Urbani strain (Figure 7, GenBank accession # AY278741). In another embodiment, the gRNA is of the polyprotein 1ab gene nucleotides from about 250 to about 21470 of the CUHK strain (Figure 9, GenBank accession # AY278554). In a further embodiment, the gRNA is of the polyprotein 1ab gene nucleotides from about 186 to about 1706 (GenBank accession # AH012999, Figure 11) from about 1 to about 10,546 (GenBank accession # AH012999, Figure 12), from about 186 to about 1,706 (GenBank accession # AY322205, Figure 20), and from about 1 to about 10,546 (GenBank accession # AY322206, Figure 21) of the Shanghai LY strain. In an alternative embodiment, the gRNA is of the polyprotein 1ab gene nucleotides from about 1 to about 3536 (GenBank accession # AH013000, Figure 14), from about 1 to about 5262 (GenBank accession # AH013000, Figure 15), and from about 1 to about 3,536 (GenBank accession # AY322197, Figure 18) of the Shanghai QXC strain.

In another embodiment, the gRNA is at least a portion of the Polyprotein 1b (also referred to as Polyprotein 1b) gene. As disclosed herein, the inventors selected an exemplary sequence (tgctaactacattttctggagg) (SEQ ID NO:71) in Polypeptide-1b to favor conditions for the exemplary multiplex RT-PCR reaction.

iii. Detecting nucleic acids, proteins, and virions

Methods for detecting RNA (such as gRNA and sgRNA) are known in the art, and include, but are not limited to, Northern blot, ribonuclease protection assay, and polymerase chain reaction.

In one embodiment, RNA (such as gRNA and sgRNA) is detected by Northern blot. The term "Northern blot" as used herein refers to the analysis of RNA by electrophoresis of RNA on agarose gels to fractionate the RNA according to size followed by transfer of the RNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized RNA is then probed with a labeled oligo-deoxyribonucleotide probe or DNA probe to detect RNA species complementary to the probe used. Northern blots provide information on both size and abundance of target RNA species. Northern blots are a standard tool of molecular biologists (J. Sambrook, *et al.* "Molecular Cloning: A Laboratory Manual," Third Edition, Publ. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

In another embodiment, RNA (such as gRNA and sgRNA) is detected by ribonuclease protection assay. Ribonuclease protection assays are used to measure the abundance of specific RNAs and to map their topological features. The method involves hybridization of test samples to complementary radiolabeled RNA probes (riboprobes), followed by digestion of non-hybridized sequences with one or more single-strand-specific ribonucleases. At the end of the digestion, the ribonucleases are inactivated, and the protected fragments of radiolabeled RNA are analyzed by polyacrylamide gel electrophoresis and autoradiography. The ribonuclease protection assay is more sensitive than the northern blot. The method can detect several target species simultaneously, and because the intensity of the signal is directly proportional to the concentration of target RNA, comparisons of the level of expression of the target gene in different tissues can be accomplished. Methods for ribonuclease protection assay are standard in the art (J. Sambrook, *et al., supra*).

In a further embodiment, RNA (such as gRNA and sgRNA) is detected by amplification of a target RNA sequence using reverse transcriptase polymerase chain reaction. The term "amplification" is defined as the production of additional copies of a nucleic acid sequence. The terms "reverse transcription polymerase chain reaction" and "RT-PCR" refer to a method for reverse transcription of an RNA sequence to generate a mixture of cDNA sequences, followed by increasing the concentration of a desired segment of the transcribed cDNA sequences in the mixture without cloning or purification. Typically, RNA is reverse transcribed using one or two primers prior to PCR amplification of the desired segment of the transcribed DNA using two primers.

Polymerase chain reaction technologies are well known in the art (Dieffenbach CW and GS Dveksler (1995) *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview NY). PCR describes a method for increasing the concentration of a segment of a target sequence in a mixture of DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme

conjugate detection; and/or incorporation of ^{32}P-labeled deoxyribonucleotide triphosphates, such as dCTP or dATP, into the amplified segment).

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer is selected such that it is sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. Suitable lengths of the primers may be empirically determined and depend on factors such as temperature, source of primer and the use of the method. In one embodiment, the primers may be from 3 to 100, preferably from 3 to 50, more preferably from 3 to 25 nucleotide bases in length.

As used herein, the terms "PCR product" and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

In one embodiment of the invention using RT-PCR, the inventors designed oligonucleotide RT-PCR primers that will amplify genomic RNA or the sgRNA that is specific to the leader-body junction and virus replication. The inventors probed for sgRNA since the presence of genomic RNA alone could result from residual input virus, while the presence of newly synthesized subgenomic RNA is indicative of virus entry and replication initiation. Genomic RNA was detected by amplifying a region between the 1b coding region and the sequence encoding the Spike (S) glycoprotein. Subgenomic RNA was detected using a primer specific to the leader sequence in conjunction with the reverse primer in S that is used for the genomic RNA detection. This procedure could be modified for any sgRNA and sensitivity could be increased by utilizing 3' genes (e.g. Nucleocapsid). However, the inventors decided to use primers specific for S because it was their opinion that this gene clearly differentiates genomic and subgenomic RNA molecules and it decreases false positives that result from viral sgRNA packaging. The SARS-CoV primer

sets were multiplexed with primers for glyceraldehyde 3' phosphate dehydrogenase (G3PDH). These primers were designed to amplify G3PDH from multiple species to serve as a positive control for RNA integrity and cDNA production. A one step RT-PCR procedure (Qiagen) was chosen to increase sensitivity over two step procedures because both "forward" and "reverse" primers can serve as reverse transcription primers of antisense and sense coronavirus RNAs, respectively. The reaction conditions (temperatures, MgCl concentrations etc.) for the multiplexed assay were optimized using SARS-CoV infected Vero E6 cells.

The sensitivity of an exemplary RT-PCR assay of SARS-CoV sgRNA was determined by analyzing RNA isolated from Vero E6 cells inoculated with serial 10-fold dilutions of SARS-CoV. Vero E6 cells were inoculated with input multiplicities of infection (MOI) ranging from 10^{-1} to 10^{-9} or were mock inoculated. Total RNA was isolated and subjected to multiplex RT-PCR at 1h and 24 h post inoculation (Figure 2). Input genomic RNA was detected at 1 PFU per 10,000 cells (Figure 2, 1 hour panel). Newly synthesized gRNA and sgRNA was detectable at 1 PFU/ million cells (Figure 2, 24 hour panel).

In any of the methods of the invention that employ detection of SARS-CoV gRNA and/or sgRNA, it may be desirable to use a negative control. Data herein shows that exemplary negative control cells include baby hamster kidney cells (BHK-21) (Figure 2A), MRC-5, MDCK, AK-D, L2, and HRT-18 cells (Figure 3) which did not produce either gRNA or sgRNA following infection with SARS-CoV.

In another embodiment, the invention's methods may employ detecting one or more SARS-coronavirus polypeptide (such as an antigen). The polypeptides may be detected by methods known in the art, such as Western blot. The terms "Western blot," "Western immunoblot," "immunoblot," and "Western" refer to the immunological analysis of protein(s), polypeptides or peptides that have been immobilized onto a membrane support. The proteins are first resolved by acrylamide gel electrophoresis to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to an antibody having reactivity towards an antigen of interest. The binding of the antibody (*i.e.*, the primary antibody) is detected by use of a secondary antibody, which specifically binds the primary antibody. The secondary antibody is typically conjugated to an enzyme, which permits visualization by the production of a colored reaction product or catalyzes a luminescent enzymatic reaction (*e.g.*, ECL reagent, Amersham). The SARS-CoV polypeptides (such as

antigens) may also be detected using enzyme-linked immunosorbent assay (ELISA), enzyme-based histochemical assays, using fluorescent, radioactive, and/or luminescent systems.

In yet another embodiment, the invention's methods employ detecting the production of SARS-coronavirus virions directly or indirectly by using, for example, electron microscopy, CPE, and infection of cells (as disclosed herein).

G. Detecting Replication of SARS-CoV Using The Invention's Exemplary Cells

The invention provides methods for detecting the presence of SARS-coronavirus in a sample, comprising: a) providing: (i) a sample; and (ii) cells chosen from one or more of the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK; b) inoculating the cells with the sample to produce inoculated cells; and c) detecting the presence of the SARS-coronavirus in the inoculated cells. These methods are useful in, for example, diagnosing the presence of SARS-CoV in samples, screening agents for their activity in reducing SARS-CoV infection, determining the relative efficacy of agents and/or modalities of treatment in altering (e.g., increasing or reducing) the levels SARS infection.

i. Cultures Containing the Invention's Cells

In one embodiment, any of the invention's methods may be performed using single cell type culture. The term "single-cell type culture" refers to a composition, whether liquid, gel, or solid, which contains one cell type (for example, HEK-293T alone, Huh-7 alone, Mv1Lu alone, pRHMK alone, or pCMK alone).

The invention further employs mixed cell type cultures. As used herein, the term "mixed-cell type culture" refers to a composition, whether liquid, gel, or solid, which contains a mixture of two or more types of cells wherein the cell types are mingled together. For example, a mixed-cell type culture may contain cells from different tissues or organs from the same species and same genus. Alternatively, a mixed-cell type culture may contain cells from different species in the same genus. Yet another alternative is that a mixed-cell type culture contains cells from a different genus. The present invention encompasses any combination of cell types. Such combinations may be suitable in, for example, the detection, identification, and/or quantitation of viruses in samples, including mixed cell cultures in which all of the cell types used are not genetically engineered,

mixtures in which one or more of the cell types are genetically engineered and the remaining cell types are not genetically engineered, and mixtures in which all of the cell types are genetically engineered.

The term "cell type different from a specifically identified cell type" as used herein, means any cell type that differs in any way from the specifically identified cell type. This term includes, without limitation, the parental cells from which the specifically identified cell type has been established (e.g., by serial culture, transfection with one or more nucleotide sequences of interest, immortalization, etc.).

An advantage of using one or more of the invention's cells (such as the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) in mixed-cell-type culture with each other is that they may provide different SARS-CoV antigens that may be used for vaccine and/or antibody production. An advantage of using one or more of the invention's cells (such as the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) in mixed-cell-type culture with other cell types, is that such cultures provide rapid and sensitive assay systems in a single unit for the detection of multiple viruses, and they also eliminate the need for multiple cell lines cultured in individual containers.

In one embodiment, the mixed cell type culture contains one or more of the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK cell together. In another embodiment, the mixed cell type culture contains one or more of the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK cell together, as well as other cell types. These mixed cell type cultures are useful in, for example, detecting the presence of viruses other than SARS-CoV. For example, mixed cell type cultures containing Mv1Lu cells and A549 cells (ATCC No. CCL185) may be used for detection of SARS-CoV, parainfluenza viruses, and influenza viruses by Mv1Lu cells, as well as detection of Herpes viruses, enteroviruses, adenoviruses, myxoviruses, and paramyxoviruses by A549 cells. Mixed cell cultures of Mv1Lu and A549 are known in the art (sold as "R-MIX™"By Diagnostic Hybrids Inc., Ohio) (U.S. Patent No. 6,376,172, incorporated by reference in its entirety).

While not limiting the invention to any particular cell type, exemplary cell lines which may be used in mixed-cell type cultures with each other and/or with any one or more of the invention's cells (such as the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) are listed in Table 2.

Table 2. Exemplary Cell Lines for Mixed-Cell Type Cultures With Cells of the Present Invention

Cell Line	ATCC #	Source	Virus[a]
1° monkey	none[b]	Kidney, rhesus monkey	Herpes, entero, adeno, myxo, paramy
BS-C-1	CCL26	Kidney, African green monkey	Herpes, entero, adeno, myxo, paramy
CV-1	CCL70	Kidney, African green monkey	Herpes, entero, adeno, myxo, paramy
Vero	CCL81	Kidney, African green monkey	Herpes, entero, adeno, myxo, paramy
Vero 76	CRL1587	Kidney, African green monkey	Herpes, entero, adeno, myxo, paramy
Vero C1008	CRL1586	Kidney, African green monkey	Herpes, entero, adeno, myxo, paramy
Vero 76	CCL81	Kidney, African green monkey	Herpes, entero, adeno, myxo, paramy
Cos-1	CRL1650	Kidney, African green monkey, transformed	Herpes, entero, adeno, myxo, paramy
Cos-7	CRL1651	Kidney, African green monkey, transformed	Herpes, entero, adeno, myxo, paramy
FRhK-4	CRL1688	Kidney, fetal rhesus monkey	Herpes, entero, adeno, myxo, paramy
LLC-MK2 original	CCL7	Kidney, rhesus monkey	Herpes, entero, adeno, myxo, paramy
LLC-MK2 derivative	CCL7.1	Kidney, rhesus monkey	Herpes, entero, adeno, myxo, paramy

Cell Line	ATCC #	Source	Virus[(a)]
MDCK	CCL34	Kidney, canine	Herpes, entero, adeno, myxo, paramy
CCD-13 Lu	CCL200	Lung, human	Herpes, entero, adeno, paramy
CCD-8 Lu	CCL201	Lung, human	Herpes, entero, adeno, paramy
CCD-14 Br	CCL203	Bronchiole, human	Herpes, entero, adeno, myxo, paramy
CCD-16 Lu	CCL204	Lung, human	Herpes, entero, adeno, paramy
CCD-18 Lu	CCL205	Lung, human	Herpes, entero, adeno, paramy
CCD-19 Lu	CCL210	Lung, human	Herpes, entero, adeno, paramy
Hs888 Lu	CCL211	Lung, human	Herpes, entero, adeno, paramy
MRC-9	CCL212	Lung, human	Herpes, entero, adeno, paramy
CCD-25 Lu	CCL215	Lung, human	Herpes, entero, adeno, paramy
WiDr	CCL218	Colon, adenocarcinoma, human	Herpes, entero, adeno
DLD-1	CCL221	Colon, adenocarcinoma, human	Herpes, entero, adeno
COLO205	CCL222	Colon, adenocarcinoma, human	Herpes, entero, adeno

Cell Line	ATCC #	Source	Virus[a]
HCT-15	CCL222	Colon, adenocarcinoma, human	Herpes, entero, adeno
SW 480	CCL228	Colon, adenocarcinoma, human	Herpes, entero, adeno
LOVO	CCL229	Colon, adenocarcinoma, human	Herpes, entero, adeno
SW403	CCL230	Colon, adenocarcinoma, human	Herpes, entero, adeno
SW48	CCL231	Colon, adenocarcinoma, human	Herpes, entero, adeno
SW116	CCL233	Colon, adenocarcinoma, human	Herpes, entero, adeno
SW1463	CCL234	Colon, adenocarcinoma, human	Herpes, entero, adeno
SW837	CCL235	Rectum, adenocarcinoma, human	Herpes, entero, adeno
SW948	CCL237	Colon, adenocarcinoma, human	Herpes, entero, adeno
SW1417	CCL238	Colon, adenocarcinoma, human	Herpes, entero, adeno
FHs74 Int	CCL241	Small intestine, adenocarcinoma, human	Herpes, entero, adeno
HCT-8	CCL244	Adenocarcinoma, ileococal	Herpes, entero, adeno
HCT-116	CCL247	Colon carcinoma, human	Herpes, entero, adeno
T84	CCL248	Colon carcinoma, human	Herpes, entero, adeno

Cell Line	ATCC #	Source	Virus[a]
NCI-H747	CCL252	Cecum, adenocarcinoma, human	Herpes, entero, adeno
NCI-H508	CCL253	Cecum, adenocarcinoma, human	Herpes, entero, adeno
LS123	CCL255	Colon, human, adenocarcinoma	Herpes, entero, adeno
CaCo-2	HTB37	Colon, adenocarcinoma, human	Herpes, entero, adeno
HT-29	HTB38	Colon, adenocarcinoma, human	Herpes, entero, adeno
SK-CO-1	HTB39	Colon, adenocarcinoma, human	Herpes, entero, adeno
HuTu 80	HTB40	Duodenum, adenocarcinoma, human	Herpes, entero, adeno
A253	HTB41	Epidemoid carcinoma	Herpes, entero, adeno, paramyo
A704	HTB45	Kidney adenocarcinoma, human	Herpes, entero, adeno, paramy
Hela	CCL2	Epitheloid carcinoma, cervix, human	Herpes, entero, adeno, myxo, paramy
Hela	CCL2.1	Epitheloid carcinoma, cervix, human	Herpes, entero, adeno, myxo, paramy
Hela53	CCL2.2	Epitheloid carcinoma, cervix, human	Herpes, entero, adeno, myxo, paramy
L-132	CCL5	Embryonic lung, human, Hela marker	Herpes, entero, adeno, myxo, paramy

Cell Line	ATCC #	Source	Virus[a]
Intestine	CCL6	Embryonic intestine, human, Hela marker	Herpes, entero, adeno
BHK-21	CCL10	Kidney, synister or golden hamster	Herpes, entero, adeno, myxo, paramy
Hak	CCL15	Kidney, synister hamster	Herpes, entero, adeno, myxo, paramy
KB	CCL17	Epidermoid carcinoma oral, human	Herpes, entero, adeno, paramy
Hep-2	CCL23	Epidermoid carcinoma larynx, human	Herpes, entero, adeno, paramy
Wish	CCL25	Amnion, human	Herpes, entero, adeno
Detroit 532	CCL54	Skin, human	Herpes, entero, adeno
FL	CCL62	Amnion, human	Herpes, entero
Detroit 525	CCL65	Skin, human	Herpes, entero, adeno
Detroit 529	CCL66	Skin, human	Herpes, entero, adeno
Detroit 510	CCL72	Skin, human	Herpes, entero, adeno
WI-38	CCL75	Lung, diploid human	Herpes, entero, adeno, paramy
WI-38 VA13	CCL75.1	Lung, diploid human, SV-40 transformed	Herpes, entero, adeno, paramy
Citrullinemia	CCL76	Skin, human	Herpes, entero, adeno, paramy
Spik (NBL-10)	CCL78	Kidney, dolphin	Herpes, entero, adeno
Detroit 539	CCL84	Skin, human	Herpes, entero, adeno
Cridu Chat	CCL90	Skin, human	Herpes, entero, adeno

Cell Line	ATCC #	Source	Virus[a]
WI26 VA4	CCL95.1	Lung, human	Herpes, entero, adeno, paramy
BeWo	CCL98	Choriocarcinoma, human	Herpes, entero, adeno
SW-13	CCL105	Adenocarcinoma, human, adrenal cortex	Herpes, entero, adeno
Detroit 548	CCL116	Skin	Herpes, entero, adeno
Detroit 573	CCL117	Skin	Herpes, entero, adeno
HT-1080	CCL121	Fibrocarcinoma, human	Herpes, entero, adeno
HG 261	CCL122	Skin, human	Herpes, entero, adeno
C211	CCL123	Skin, human	Herpes, entero, adeno
Amdur II	CCL124	Skin, human	Herpes, entero, adeno
CHP 3 (M.W.)	CCL132	Skin, human, fibroid like	Herpes, entero, adeno
CHP 4 (W.W.)	CCL133	Skin, human, fibroid like	Herpes, entero, adeno
RD	CCL136	Rhabdomyosarcoma	Herpes, entero, adeno
HEL 299	CCL137	Lung, diploid	Herpes, entero, adeno, paramy
Detroit 562	CCL138	Carcinoma, pharynx	Herpes, entero, adeno, myxo, paramy
MRC-5	CCL171	Lung, diploid, human	Herpes, entero, adeno, paramy
A-549	CCL185	Lung, carcinoma, human	Herpes, entero, adeno, myxo, paramy
IMR-90	CCL186	Lung, carcinoma, human	Herpes, entero, adeno, myxo, paramy

Cell Line	ATCC #	Source	Virus[a]
LS180	CCL187	Colon, adenocarcinoma, human	Herpes, entero, adeno
LS174T	CCL188	Colon, adenocarcinoma, human	Herpes, entero, adeno
NCI-H292	CCL-1848	Mucoepidermoid, human	Respir. syncytial virus
BHK/ICP6LacZ-5	CCL-12072		
CV-1	CCL-70		
hs27	HFF; CRL-1634		
Mv1Lu	CCL-64		
McCoy	CCL-1696		
MRC-5	CCL-171		
Vero	CCL-81		
MDCK (NBL-2)	CCL-34		
BHK21	CCL-10		
Mv1Lu-hF	PTA-4737	Lung, epithelial, mink	Influenza, parainfluenza

[a] Herpes = Herpes viruses; Entero = Enteroviruses; Adeno = Adenoviruses; Myxo = Myxoviruses; and Paramy = Paramyxoviruses.

[b] Primary monkey kidney cells may be obtained from Diagnostic Hybrids (catalog numbers 490102A for shell format and 49-0600A for tube format).

In one embodiment, it may be desirable use Mv1Lu cells for the replication and/or detection of parainfluenza and influenza viruses without replication and/or detection of SARS-CoV. This is advantageous in laboratories that diagnose infection with parainfluenza

and influenza viruses, and that do not have access to containment facilities that are required for manipulation of SARS-CoV. In one embodiment, this goal may be achieved by incubating a test sample with Mv1Lu cells for up to 24 hours. This is based on data herein (Figure 4B), which shows that SARS-CoV was not produced by Mv1Lu cells within 24 hours p.i. In another embodiment, this goal may be achieved by contacting one or more of the Mv1Lu cells and the sample with antibody specific for one or more SARS-coronavirus antigen.

In a further embodiment, the goal of reducing infection of Mv1Lu cells by SARS-coronavirus, while not substantially reducing susceptibility of Mv1Lu cells to parainfluenza and/or influenza viruses, may be attained by contacting the Mv1Lu cells and/or sample that is being tested with a protease inhibitor, as further described below.

In another embodiment, it may be desirable to use Mv1Lu cells for the detection and/or proliferating of parainfluenza and influenza viruses in addition to detection and/or replication of SARS-CoV, such as where the specificity of action of certain reagents on different viruses is being investigated. This may be achieved by incubating Mv1Lu cells with a test sample for more than 24 hours (Figure 4B).

ii. Cells frozen *in situ*

In one embodiment, the invention's cells (such as the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) are frozen *in situ*. Methods for the *in situ* growth, freezing and testing of cultured cells are known in the art (Patent No. 6,472,206, incorporated by reference in its entirety). In one embodiment, the in situ frozen cells are in single cell type culture. In another embodiment, the in situ frozen cells are in mixed cell type culture.

iii. Samples

The invention contemplates contacting the invention's cells (such as the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) with a sample for the purpose of, for example and without limitation, detecting and/or quantitating SARS-CoV polypeptides, proteins, and/or virus particles in a sample. The terms "sample" and "specimen" as used herein are used in their broadest sense to include any composition that is obtained and/or derived from biological or environmental source, as well as sampling devices (*e.g.*, swabs), which are brought into contact with biological or environmental

samples. "Biological samples" include those obtained from an animal (including humans, domestic animals, as well as feral or wild animals, such as ungulates, bear, fish, lagamorphs, rodents, *etc.*), body fluids such as urine, blood, plasma, fecal matter, cerebrospinal fluid (CSF), semen, sputum, and saliva, as well as solid tissue. Biological samples also include a cell (such as cell lines, cells isolated from tissue whether or not the isolated cells are cultured after isolation from tissue, fixed cells such as cells fixed for histological and/or immunohistochemical analysis), tissue (such as biopsy material), cell extract, tissue extract, and nucleic acid (*e.g.,* DNA and RNA) isolated from a cell and/or tissue, and the like. Also included are materials obtained from food products and food ingredients such as dairy items, vegetables, meat, meat by-products, and waste. Environmental samples" include environmental material such as surface matter, soil, water, and industrial materials, as well as material obtained from food and dairy processing instruments, apparatus, equipment, disposable, and non-disposable items. In one embodiment, the biological sample is a cell, tissue, and or fluid obtained from a mammal, including from the upper respiratory tissues (such as nasopharyngeal wash, nasopharyngeal aspirate, nasopharyngeal swab, and oropharyngeal swab), from the lower respiratory tissues (such as bronchiolar lavage, tracheal aspirate, pleural tap, sputum), blood, plasma, serum, stool, and tissue from any organ such as, without limitation, lung, heart, spleen, liver, brain, kidney, and adrenal glands. These examples are illustrative, and are not to be construed as limiting the sample types applicable to the present invention.

While not intending to limit the source of the sample, in one embodiment, the sample is isolated from a mammal. In one embodiment, the "mammal" is rodent (such as mouse and rat, such as cotton rat), primate (including simian and human) ovine, bovine, ruminant, lagomorph, porcine, caprine, equine, canine, feline, avian, *etc.* Expressly included are hamster, mink, ferret, pig, cat, and rabbit.

The invention also provides methods for detecting the presence of SARS-CoV in one or more samples, such as in samples from mammals that have been treated with anti-SARS-CoV agents. These methods may be used in, for example, determining the efficacy of a therapeutic modality (such as a chemical drug) in reducing SARS-coronavirus infection in a mammal, including a model animal and human. These methods are also useful in determining the relative efficacy of different therapeutic modalities, such as different concentrations of the same drug, the same concentration of different drugs, and different combinations of drugs.

Thus, in one embodiment, the invention provides a method for detecting the presence of SARS-coronavirus in a first sample and in a second sample, comprising: a) providing: (i) a first sample; (ii) a second sample; b) contacting test cells chosen from one or more of the invention's cells (such as the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) with: (i) the first sample to produce a first treated sample; and (ii) the second sample to produce a second treated sample; wherein the exposing is under conditions such that the test cells are infected with SARS-coronavirus; c) detecting the presence of one or more of SARS-coronavirus gRNA and SARS-coronavirus sgRNA, wherein the detecting indicates the presence of the SARS-coronavirus.

In a preferred embodiment, the detecting step comprises detecting one or more of: i) absence of SARS-coronavirus gRNA in the first treated sample; ii) reduced level of SARS-coronavirus sgRNA in the first treated sample compared to the level of sgRNA in the second treated sample; and iii) reduced ratio of SARS-coronavirus sgRNA level to SARS-coronavirus gRNA level in the first treated sample compared to in the second treated sample. Detection of any one or more of these phenomena indicates that the first sample contains a reduced level of SARS-coronavirus compared to the second sample.

In one embodiment, the method comprises detecting an absence of SARS-coronavirus gRNA in the first treated sample. Without limiting the invention to any particular mechanism, such detection indicates that SARS-coronavirus has not adsorbed to cells from which the first sample was obtained.

In another embodiment, the method comprises detecting an absence of SARS-coronavirus sgRNA in the first treated sample. Without limiting the invention to any particular mechanism, such detection indicates that SARS-coronavirus has not replicated in cells from which the first sample was obtained.

In a preferred embodiment, the method comprises detecting an absence of SARS-coronavirus gRNA and SARS-coronavirus sgRNA in the first treated sample. Without limiting the invention to any particular mechanism, such detection indicates that SARS-coronavirus has neither adsorbed to nor replicated in cells from which the first sample was obtained.

In one embodiment, the first sample and the second sample are from a mammal. In a preferred embodiment, the first sample is from a mammal treated with an agent and the second sample is from the mammal that is not treated with the agent. These steps may be

used in, for example, identifying an agent as reducing infection with SARS-CoV in a model animal or in human clinical trials.

In another embodiment, the first sample is from a mammal treated with a first concentration of an agent and the second sample is from the mammal treated with a second concentration of the agent, wherein the first and second concentrations are different. These steps may be used in, for example, comparing the relative efficacy of different concentrations of the same agent in reducing infection with SARS-CoV in a model animal or in human clinical trials.

In a further embodiment, the first sample is from a mammal treated with a first agent and the second sample is from the mammal treated with a second agent wherein the first and second agents are different. These steps may be used in, for example, comparing the relative efficacy of different agents in reducing infection with SARS-CoV in a model animal or in human clinical trials.

H. Screening Anti-SARS-CoV Agents

In one embodiment, the invention provides a method for identifying a test agent as altering infection of a cell by SARS-coronavirus, comprising: a) providing cells treated with a test agent, wherein the cells are chosen from one or more of HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK; and b) detecting an altered level of infection of cells treated with the test agent compared to a level of infection of the cells not treated with the test agent, wherein the detecting identifies the test agent as altering infection of a cell by SARS-coronavirus. The altered level of infection may be a reduced level or an increased level.

This method may be used in, for example, screening anti-SARS-coronavirus drugs. Anti-SARS-coronavirus drugs may be used as prophylactic agents and/or therapeutic agents in the treatment of SARS-coronavirus. Anti-SARS-coronavirus drugs may also be used to increase the safety of handling cells, such as Mv1Lu cells that are used in clinical laboratories for and that may be susceptible and/or permissive to SARS-coronavirus. For example, with respect to Mv1Lu cells, which are routinely used in clinical laboratories for screening infection with influenza and parainfluenza viruses, and which show low permissivity to SARS-CoV, particularly useful are anti-SARS CoV drugs that reduce permissivity of Mv1Lu cells to SARS, while not substantially reducing susceptibility and/or permissivity of Mv1Lu cells to influenza virus and/or parainfluenza virus. The invention's

methods are also useful in determining the efficacy of a drug in reducing infection in a model mammal and in human clinical trials.

In one embodiment, the detecting step may comprise detecting SARS-coronavirus sgRNA, gRNA, polypeptide and/or virion. In another embodiment, the detecting step comprises detecting one or more of: i) absence of SARS-coronavirus gRNA in the treated cells; ii) reduced level of SARS-coronavirus sgRNA in the treated cells compared to the level of sgRNA in the cells that are not treated with the test agent; and (iii) reduced ratio of SARS-coronavirus sgRNA level to SARS-coronavirus gRNA level in the treated cells compared to in the cells that are not treated with the test agent; wherein the detecting identifies the test agent as reducing infection of a cell by SARS-coronavirus.

In another embodiment, it may be desirable to compare the efficacy of two agents in reducing infection with SARS-coronavirus. This may be achieved by detecting one or more of: i) reduced level of SARS-coronavirus sgRNA in the cells treated with a second test agent compared to the level of sgRNA in the cells treated with the test agent; and ii) reduced ratio of SARS-coronavirus sgRNA level to SARS-coronavirus gRNA level in the cells treated with a second test agent compared to the ratio in the cells treated with the test agent, wherein detecting an increased reduction in one or more of the level of SARS-coronavirus sgRNA and of the ratio of SARS-coronavirus sgRNA level to SARS-coronavirus gRNA level in the cells treated with the test agent compared to the cells treated with the second test agent identifies the test agent as more efficacious than the second test agent in reducing infection of a cell by SARS-coronavirus.

The "agent" identified by, and/or used by, the invention's methods refers to any type of molecule (for example, a peptide, nucleic acid, carbohydrate, lipid, organic, and inorganic molecule, *etc.*) obtained from any source (for example, plant, animal, and environmental source, *etc.*), or prepared by any method (for example, purification of naturally occurring molecules, chemical synthesis, and genetic engineering methods, *etc.*). The terms "test compound," "compound," "agent," "test agent," "molecule," and "test molecule," as used herein, refer to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function. Agents comprise both known and potential therapeutic compounds. An agent can be determined to be therapeutic by screening using the screening methods of the present invention. A "known therapeutic compound" refers to a therapeutic compound that has been shown (e.g., through animal trials or prior experience with administration to humans)

to be effective in such treatment or prevention. In other words, a known therapeutic compound is not limited to a compound efficacious in the treatment of SARS-coronavirus infection. Agents are exemplified by, but not limited to, vaccines, antibodies, nucleic acid sequences such as ribozyme sequences, and other agents as further described herein.

In one embodiment, the agent is an antibody that is specific for one or more SARS-coronavirus antigens. The terms "antibody" and "immunoglobulin" are interchangeably used to refer to a glycoprotein or a portion thereof (including single chain antibodies), which is evoked in an animal by an immunogen and which demonstrates specificity to the immunogen, or, more specifically, to one or more epitopes contained in the immunogen. The term "antibody" includes polyclonal antibodies, monoclonal antibodies, naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof, including, for example, Fab, $F(ab')_2$, Fab fragments, Fd fragments, and Ev fragments of an antibody, as well as a Fab expression library. It is intended that the term "antibody" encompass any immunoglobulin (*e.g.*, IgG, IgM, IgA, IgE, IgD, *etc.*) obtained from any source (*e.g.*, humans, rodents, non-human primates, caprines, bovines, equines, ovines, *etc.*). The term "polyclonal antibody" refers to an immunoglobulin produced from more than a single clone of plasma cells; in contrast "monoclonal antibody" refers to an immunoglobulin produced from a single clone of plasma cells. Monoclonal and polyclonal antibodies may or may not be purified. For example, polyclonal antibodies contained in crude antiserum may be used in this unpurified state.

Naturally occurring antibodies may be generated in any species including, for example, murine, rat, rabbit, hamster, human, and simian species using methods known in the art. Non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as previously described (Huse *et al.*, Science 246:1275-1281 (1989)). These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward *et al.*, Nature 341:544-546 (1989); Hilyard *et al.*, Protein Engineering: A practical approach (IRL Press 1992); and Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995).

Those skilled in the art know how to make polyclonal and monoclonal antibodies, which are specific to a desirable polypeptide. For the production of monoclonal and polyclonal antibodies, various host animals can be immunized by injection with the peptide corresponding to any molecule of interest in the present invention, including but not limited to rabbits, mice, rats, sheep, goats, *etc.* In one embodiment, the peptide is conjugated to an immunogenic carrier (*e.g.,* diphtheria toxoid, bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH)). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active molecules such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and *Corynebacterium parvum.*

For preparation of monoclonal antibodies directed toward molecules of interest in the present invention, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used (*See, e.g.,* Harlow and Lane, *Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, NY). These include but are not limited to the hybridoma technique originally developed by Köhler and Milstein (Köhler and Milstein, Nature 256:495-497 (1975)), as well as the trioma technique, the human B-cell hybridoma technique (*See e.g.,* Kozbor *et al.* Immunol. Today 4:72, 1983), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al., in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96, 1985). In one embodiment of the monoclonal antibodies are of the IgG class.

In additional embodiments of the invention, monoclonal antibodies can be produced in germ-free animals utilizing technology such as that described in PCT/US90/02545. In addition, human antibodies may be used and can be obtained by using human hybridomas (Cote *et al.*, Proc. Natl. Acad. Sci. U.S.A.80:2026-2030 (1983)) or by transforming human B cells with EBV virus in vitro (Cole *et al.*, *in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96 (1985)).

Furthermore, techniques described for the production of single chain antibodies (*See e.g.,* U.S. Patent 4,946,778; herein incorporated by reference) can be adapted to produce single chain antibodies that specifically recognize one or more SARS antigens. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse *et al.,* Science 246:1275-1281 (1989)) to

allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a particular protein or epitope of interest (*e.g.*, at least a portion of an AUBP or mammalian exosome).

The invention also contemplates humanized antibodies. Humanized antibodies may be generated using methods known in the art, including those described in U.S. Patent Numbers 5,545,806; 5,569,825 and 5,625,126, the entire contents of which are incorporated by reference. Such methods include, for example, generation of transgenic non-human animals which contain human immunoglobulin chain genes and which are capable of expressing these genes to produce a repertoire of antibodies of various isotypes encoded by the human immunoglobulin genes.

According to the invention, techniques described for the production of single chain antibodies (U.S. 4,946,778; herein incorporated by reference) can be adapted to produce specific single chain antibodies as desired. An additional embodiment of the invention utilizes the techniques known in the art for the construction of Fab expression libraries (Huse et al., Science, 246:1275-1281 (1989)) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibody fragments that contain the idiotype (antigen binding region) of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragment that can be produced by pepsin digestion of an antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of an F(ab')2 fragment, and the Fab fragments that can be generated by treating an antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art (*e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (*e.g.*, using colloidal gold, enzyme or radioisotope labels), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays, *etc.*), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, *etc.*

In an alternative embodiment, the agent is a nucleic acid sequence. The terms "nucleic acid sequence" and "nucleotide sequence" as used herein refer to two or more nucleotides, which are covalently linked to each other. Included within this definition are

oligonucleotides, polynucleotide, and fragments or portions thereof, DNA or RNA of genomic or synthetic origin, which may be single- or double-stranded, and represent the sense or antisense strand. Nucleic acid sequences, which are particularly useful in the instant invention include, without limitation, antisense sequences and ribozymes.

In one embodiment, the agent that alters the infection by SARS-coronavirus is an antisense nucleic acid sequence, which hybridizes with at least a portion of SARS-coronavirus genomic RNA and/or subgenomic RNA. Antisense sequences have been successfully used to inhibit the expression of several genes (Markus-Sekura (1988) Anal. Biochem. 172:289-295; Hambor *et al.* (1988) J. Exp. Med. 168:1237-1245; and patent EP 140 308), including the gene encoding VCAM1, one of the integrin $\alpha 4\beta 1$ ligands (U.S. Patent No. 6,252,043, incorporated in its entirety by reference). The terms "antisense DNA sequence" and "antisense sequence" as used herein interchangeably refer to a deoxyribonucleotide sequence whose sequence of deoxyribonucleotide residues is in reverse 5' to 3' orientation in relation to the sequence of deoxyribonucleotide residues in a sense strand of a DNA duplex. A "sense strand" of a DNA duplex refers to a strand in a DNA duplex which is transcribed by a cell in its natural state into a "sense mRNA." Sense mRNA generally is ultimately translated into a polypeptide. Thus, an "antisense DNA sequence" is a sequence which has the same sequence as the non-coding strand in a DNA duplex, and which encodes an "antisense RNA" (*i.e.*, a ribonucleotide sequence whose sequence is complementary to a "sense mRNA" sequence). The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.*, "positive") strand. Antisense RNA may be produced by any method, including synthesis by splicing an antisense DNA sequence to a promoter, which permits the synthesis of antisense RNA. The transcribed antisense RNA strand combines with natural mRNA produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation, or promote its degradation.

Antisense oligonucleotide sequences may be synthesized using any of a number of methods known in the art (such as solid support and commercially available DNA synthesizers, standard phosphoramidate chemistry techniques, and commercially available services, *e.g.*, Genta, Inc.).

Other molecules which find use as agents for altering infection by SARS-coronavirus include organic molecules, inorganic molecules, and libraries of any type of

molecule, which can be screened using a method of the invention, and which may be prepared using methods known in the art. These agents are made by methods for preparing oligonucleotide libraries (Gold *et al.*, U.S. Patent No. 5,270,163, incorporated by reference); peptide libraries (Koivunen *et al.* J. Cell Biol., 124: 373-380 (1994)); peptidomimetic libraries (Blondelle *et al.*, Trends Anal. Chem. 14:83-92 (1995)) oligosaccharide libraries (York *et al.*, Carb. Res. 285:99-128 (1996) ; Liang *et al.*, Science 274:1520-1522 (1996); and Ding *et al.*, Adv. Expt. Med. Biol. 376:261-269 (1995)); lipoprotein libraries (de Kruif *et al.*, FEBS Lett., 399:232-236 (1996)); glycoprotein or glycolipid libraries (Karaoglu *et al.*, J. Cell Biol. 130:567-577 (1995)); or chemical libraries containing, for example, drugs or other pharmaceutical agents (Gordon *et al.*, J. Med. Chem. 37:1385-1401 (1994); Ecker and Crook, Bio/Technology 13:351-360 (1995), U.S. Patent No. 5,760,029, incorporated by reference). Libraries of diverse molecules also can be obtained from commercial sources.

I. Administering Anti-SARS-CoV Agents

The invention provides a method for reducing infection by SARS-coronavirus comprising administering a therapeutic amount of an agent to a mammal. The terms "therapeutic amount," "pharmaceutically effective amount," "therapeutically effective amount," "biologically effective amount," and are used interchangeably herein to refer to an amount which is sufficient to achieve a desired result, whether quantitative or qualitative. In particular, a pharmaceutically effective amount is that amount that results in the reduction, delay, and/or elimination of undesirable effects (such as pathological, clinical, biochemical and the like) in the subject that are associated with infection with SARS-coronavirus. As used herein, the actual amount encompassed by the term "therapeutic amount" will depend on the route of administration, the type of subject being treated, and the physical characteristics of the specific subject under consideration. These factors and their relationship to determining this amount are well known to skilled practitioners in the medical, veterinary, and other related arts. This amount and the method of administration can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors, which those skilled in the art will recognize.

In one embodiment, the agent is administered for a "therapeutically effective time" refers to the period of time during which a pharmaceutically effective amount of a compound is administered, and that is sufficient to reduce one or more symptoms associated with SARS-coronavirus infection.

The agent may be administered before, concomitantly with, and/or after detection of symptoms of infection with SARS-coronavirus. The term "concomitant" when in reference to the relationship between administration of a compound and disease symptoms means that administration occurs at the same time as, or during, manifestation of symptom associated with SARS-coronavirus infection. Also, the invention's agents may be administered before, concomitantly with, and/or after administration of another type of drug or therapeutic procedure.

As used herein, the actual amount encompassed by the term "therapeutic amount" will depend on the nature of agent, route of administration, the type of subject being treated, and the physical characteristics of the specific subject under consideration. These factors and their relationship to determining this amount are well known to skilled practitioners in the medical, veterinary, and other related arts. This amount and the method of administration can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors, which those skilled in the art will recognize. The dosage amount and frequency are selected to create an effective level of the compound without substantially harmful effects. The agent may be administered by, for example, oral, parenteral (*e.g.*, subcutaneous, intravenous, intramuscular, intrastemal injection, and infusion), intranasal, and/or inhalation routes. A therapeutic amount of the agent may be determined using *in vitro* and *in vivo* assays known in the art

The agents may be administered with one or more pharmaceutically acceptable carrier, diluent or excipient. Pharmaceutically acceptable carriers are known in the art such as those described in, for example, Remingtons Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The pharmaceutically acceptable carriers may be liquid, with the compositions being, for example, an oral syrup or injectable liquid. Compositions in solid or liquid form may include an agent, which binds to the active component(s) and thereby assists in the delivery of the active components. Suitable agents that may act in this capacity include a monoclonal or polyclonal antibody, a protein or a liposome. Alternatively, the pharmaceutical composition of the present invention may consist of gaseous dosage units, *e.g.*, it may be in the form of an aerosol useful in, for example, inhalatory administration. The term "aerosol" is used to denote a variety of systems ranging from those of colloidal nature to systems consisting of pressurized packages. Delivery may be by a liquefied or compressed gas or by a suitable pump system, which dispenses the active ingredients. Aerosols of compounds of the invention may be delivered in single

phase, bi-phasic, or tri-phasic systems in order to deliver the active ingredient(s). Delivery of the aerosol includes the necessary container, activators, valves, subcontainers, spacers and the like, which together may form a kit. Preferred aerosols may be determined by one skilled in the art, without undue experimentation.

Liquid pharmaceutical compositions of the invention, whether they be solutions, suspensions or other like form, may include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or digylcerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, cyclodextrin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

J. Producing SARS-CoV Particles and SARS-CoV Polypeptides

The invention also provides a method for producing one or more of SARS-coronavirus particles and SARS-coronavirus polypeptide, comprising: a) providing: (i) SARS-coronavirus; and (ii) a cell type chosen from one or more of HEK-293T, Huh-7, Mv1Lu, pRHMK and pCMK; and b) inoculating the cell type with the virus under conditions such that the inoculated cell produces one or more of SARS-coronavirus and SARS-coronavirus polypeptide. One advantage in using a combination of cells that are infected with SARS-CoV to generate antibodies and/or vaccines is that each cell in the combination may differently process the viral proteins. Thus, a combination of cells infected with SARS-CoV would enable the generation of antibodies and/or vaccines that are specific to different viral proteins, thereby increasing the sensitivity and/or specificity of the antibodies and/or vaccines in SARS-CoV detection and/or treatment.

In one embodiment, the invention's methods may be used to produce one or more SARS-coronavirus antigens. The terms "antigen," "immunogen," "antigenic," "immunogenic," "antigenically active," and "immunologically active" refer to any molecule that is capable of inducing a specific humoral or cell-mediated immune response. An

immunogen generally contains at least one epitope. Immunogens are exemplified by, but not restricted to molecules, which contain a peptide, polysaccharide, nucleic acid sequence, and/or lipid. Complexes of peptides with lipids, polysaccharides, or with nucleic acid sequences are also contemplated, including (without limitation) glycopeptide, lipopeptide, glycolipid, *etc.* These complexes are particularly useful immunogens where smaller molecules with few epitopes do not stimulate a satisfactory immune response by themselves.

The terms "epitope" and "antigenic determinant" refer to a structure on an antigen, which interacts with the binding site of an antibody and/or T cell receptor as a result of molecular complementarity. An epitope may compete with the intact antigen, from which it is derived, for binding to an antibody. Generally, secreted antibodies and their corresponding membrane-bound forms are capable of recognizing a wide variety of molecules as antigens, whereas T cell receptors are capable of recognizing only fragments of proteins which are complexed with MHC molecules on cell surfaces. Antigens recognized by immunoglobulin receptors on B cells are subdivided into three categories: T-cell dependent antigens, type 1 T cell-independent antigens; and type 2 T cell-independent antigens. Also, for example, when a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (*i.e.*, the immunogen used to elicit the immune response) for binding to an antibody.

Exemplary SARS-coronavirus antigens include, without limitation, at least a portion of a SARS-CoV polypeptide chosen from one or more of Nucleocapsid (N), Spike glycoprotein (S), Matrix (M), E protein, and Replicase proteins (Pol 1a/b) described *supra*.

SARS-CoV polypeptides and antigens may be made using methods known in the art. In one embodiment, SARS-CoV antigens may be obtained by purifying them using routine methods, from cells (such as the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*) that are infected with SARS-CoV.

In another embodiment, SARS-CoV polypeptides (such as antigens) may be synthesized by chemical synthesis. Synthetic chemistry techniques, such as solid phase Merrifield synthesis are advantageous for reasons of purity, freedom from undesired side products, ease of production, etc. A summary of the techniques available are found in

several articles, including Steward et al., Solid Phase Peptide Synthesis, W. H. Freeman, Co., San Francisco (1969); Bodanszky, et al., Peptide Synthesis, John Wiley and Sons, Second Edition (1976); J. Meienhofer, Hormonal Proteins and Peptides, 2:46, Academic Press (1983); Merrifield, Adv. Enzymol. 32:221-96 (1969); Fields, et al., Intl. Peptide Protein Res., 35:161-214 (1990), and U.S. Pat. No. 4,244,946 for solid phase peptide synthesis; and Schroder et al., The Peptides, Vol 1, Academic Press (New York) (1965) for classical solution synthesis. Protecting groups usable in synthesis are described as well in Protective Groups in Organic Chemistry, Plenum Press, New York (1973). Solid phase synthesis methods consist of the sequential addition of one or more amino acid residues or suitably protected amino acid residues to a growing peptide chain. Either the amino group or the carboxyl group of the first amino acid residue is protected by a suitable selectively removable protecting group. A different, selectively removable protecting group is utilized for amino acids containing a reactive side group such as lysine.

In a further embodiment, SARS-CoV polypeptides (such as antigens) may be produced by expression of recombinant DNA constructs prepared in accordance with well-known methods. Such production can be desirable to provide large quantities or alternative embodiments of such compounds. In one embodiment, DNA sequences of open reading frames (ORFs) encoding the desired peptide sequence is prepared using commercially available nucleic acid synthesis methods. The chemically synthesized DNA is isolated in a purified form, and inserted into an expression vector, as exemplified by, but not limited to, plasmid, phagemid, shuttle vector, cosmid, and virus.

Expression can be effected in prokaryotic, eukaryotic and/or viral hosts. Prokaryotes most frequently are represented by various strains of *E. coli*. However, other microbial strains may also be used, such as bacilli, for example *Bacillus subtilis*, various species of Pseudomonas, or other bacterial strains. In such prokaryotic systems, plasmid vectors, which contain replication sites and control sequences derived from a species compatible with the host are used. For example, a workhorse vector for E. coli is pBR322 and its derivatives. Commonly used prokaryotic control sequences, which contain promoters for transcription initiation, optionally with an operator, along with ribosome binding-site sequences, include such commonly used promoters as the beta-lactamase (penicillinase) and lactose (lac) promoter systems, the tryptophan (trp) promoter system, and the lambda-derived PL promoter and N-gene ribosome binding site. However, any available promoter system compatible with prokaryote expression can be used.

Expression in eukaryotic cells (such as yeast, insect, and mammalian cells) is expressly contemplated. This method is particularly suited for glycoproteins, such as S.

Expression by viral vectors may be achieved using, for example, vaccinia viruses, retroviral vectors, alpha viruses, influenza virus, adenoviruses, and baculoviruses, which may be engineered to express the SARS-CoV polypeptides and/or antigens upon infection/transduction of various cell types. These systems can be used to infect a variety of cell types from mammalian to insect cells. This approach may be very efficient resulting in very high level protein expression.

In one embodiment, the SARS-CoV polypeptide (such as antigen) may be isolated following recombinant expression. The terms "isolated," "to isolate," "isolation," "purified," "to purify," "purification," and grammatical equivalents thereof as used herein, refer to the reduction in the amount of at least one contaminant (such as protein and/or nucleic acid sequence) from a sample. Thus purification results in an "enrichment," *i.e.*, an increase in the amount of a desirable protein and/or nucleic acid sequence in the sample. For example, the SARS-CoV polypeptide (such as antigen) may be fused to another molecule capable of binding to a ligand. The ligand may be immobilized to a solid support to facilitate isolation of the fused polypeptide. Ligand-binding systems useful for the isolation of polypeptides are commercially available and include, for example, the staphylococcal protein A and its derivative ZZ (which binds to human polyclonal IgG), histidine tails (which bind to Ni^{2+}), biotin (which binds to streptavidin), maltose-binding protein (MBP) (which binds to amylose), glutathione S-transferase (which binds to glutathione), *etc*. It is not intended that the polypeptide probes of the present invention be limited to any particular isolation system. The use of 6-8 Histidine tags in combination with Ni^{2+} chromatography has been successfully used for the production of N and E proteins of other CoVs (*e.g.*, MHV).

In one embodiment, the SARS-CoV particles and/or antigens find use in antibody generation. These antibodies may be used in diagnostic assays for the detection of SARS-CoV, as described *supra*. The antibodies may also be used in the prophylaxis and/or treatment of SARS-CoV infection.

In another embodiment, the cells and methods of the invention are useful for the production of SARS-CoV particles and/or antigens for use in vaccine formulations. The term "vaccine" as used herein refers to a preparation of a pathogenic organism (such as virus as exemplified by SARS-CoV and human immunodeficiency virus, bacterium, fungus,

protist such as the malaria agent *Plasmodium*, multicellular parasite such as *Schistosoma*, *etc.*) and/or an antigen isolated from the organism, which can be administered prophylactically to an animal to induce immunity. Vaccines include, but are not limited to, live attenuated vaccines, inactivated vaccines, and subunit vaccines. Methods for making and using vaccines are known in the art (Murphy and Chanock, "Immunization against viral diseases" Chapter 16 pp. 435- 467, Eds. Knipe and Howley, Publ. Lippincott Williams and Wilkins, Philadelphia, Field's Virology Fourth Edition, 2001).

In one embodiment, the vaccine is a live attenuated vaccine. The term "live attenuated vaccine" refers to a strain (preferably an avirulent strain) of a pathogenic organism that is nonpathogenic and which still induces specific immunity against the pathogenic organism. Methods for producing live attenuated vaccines are known in the art such as those that employ vaccinia virus to vaccinate against smallpox. In one embodiment, passage of the virulent virus in cell culture can be used to produce a live attenuated vaccine strain. Such vaccines are exemplified by those for measles, mumps and rubella. In another embodiment, live attenuated vaccines may be produced by introducing site-specific mutations into virulence genes to produce an attenuated virus strain for vaccine.

The term "inactivated vaccine" refers to a preparation of a killed and/or inactivated pathogenic organism. Methods for making inactivated vaccines are known in the art such as by chemical inactivation of virus that has been grown in eggs or in cell culture. Successful inactivated vaccines have been produced for rabies and influenza. In one embodiment, inactivated SARS-CoV vaccine may be prepared from virus produced by the invention's cells (such as the exemplary HEK-293T, Huh-7, Mv1Lu, pRHMK and/or pCMK cell, *etc.*). The virus is isolated from the culture medium by affinity chromatography using cellufine sulphate (Palache *et al.*, J. Infect. Dis. 176(suppl. 1):S20-S23 (1997)). The intact live virus is inactivated after purification by any one of a number of methods known in the art, such as formalin and/or propiolactone treatment to produce an inactivated viral vaccine. Virus inactivation may be achieved by incubation of the virus suspension in 0.1% formaldehyde for 10 to 14 days at 4°C. The inactivated viral preparation is then tested in a model mammal using standard protocols, before use in human clinical trials.

The term "subunit vaccine" refers to an antigenic polypeptide of the pathogenic organism that has been recombinantly expressed *in vitro*. Methods for making subunit vaccines are known in the art such as for the hepatitis B virus vaccine, which was generated using hepatitis B surface antigen expressed in yeast cells. A "subunit vaccine" also refers to

recombinant viral or bacterial vectors that express genes encoding an antigenic polypeptide of the pathogenic organism. Exemplary recombinant viral vectors include vaccinia virus, adenovirus, paramyxoviruses, avian poxviruses, yellow fever virus and vesicular stomatitis virus. A "subunit vaccine" further includes DNA sequences, such as a plasmid containing the coding sequence for an antigen that is linked to a strong promoter sequence that is active in mammalian cells. Such plasmids are inoculated directly into the host, the viral gene is expressed in the host and antibody and cell-mediated immunity can then be induced to the recombinant antigen.

Vaccines and/or antibodies against SARS-CoV may be used for immunizing a mammal against SARS-coronavirus, by administering these compositions to generating an immune response in the mammal against SARS-coronavirus. In one embodiment, vaccines and/or antibodies are used therapeutically in a mammal that is already infected with SARS-coronavirus. In another embodiment, vaccines and/or antibodies are used prophylactically in a mammal that is not known to be infected with a SARS-coronavirus.

K. Compositions And Methods For Using Protease Inhibitors To Reduce Infection With Plus-strand RNA viruses

The invention provides compositions and methods for reducing infection with plus-strand RNA viruses. In one embodiment, the invention provides a composition comprising (i) cells susceptible to a virus that is not a plus-strand RNA virus, and (ii) protease inhibitor. The terms "positive-strand RNA virus "plus-strand RNA virus," and "+-strand RNA virus," are equivalent terms that refer to a virus whose genome contains a plus-strand RNA.

Without intending to limit the type of the virus, in one embodiment, the "virus that is not a plus-strand RNA virus" contains a genome of single stranded DNA, double stranded DNA, double stranded RNA, or negative-strand RNA. Also without limiting the source of the virus, the virus that is not a plus-strand RNA virus may be an animal virus, plant virus, and bacteriophage. More particularly, the animal virus that is not a plus-strand RNA virus is exemplified by, but not limited to, Arenaviridae, Baculoviridae, Birnaviridae, Bunyaviridae, Cardiovirus, Corticoviridae, Cystoviridae, Epstein-Barr virus, Filoviridae, Hepadnviridae, Hepatitis virus, Herpesviridae, Influenza virus, Inoviridae, Iridoviridae, Metapneumovirus, Orthomyxoviridae, Papovaviru, Paramyxoviridae, Parvoviridae, Polydnaviridae, Poxyviridae, Reoviridae, Rhabdoviridae, Semliki Forest virus, Tetraviridae, Toroviridae, Vaccinia virus, Vesicular stomatitis virus.

Cells that are susceptible to viruses are known in the art and are exemplified, but not limited to, cells susceptible to metapneumovirus, cells susceptible to cells susceptible to Arbovirus (such as BHK-21 cells), cells susceptible to BK polyomavirus (such as NCI-H292 cells), cells susceptible to BVDV (such as BT, and EBTr cells), cells susceptible to CMV (such as H&V-Mix, HEL, HEL-299, HFL-Chang, Hs27 (HFF), Human Fetal Tonsil, MRC-5, MRHF, Mv1Lu, and WI-38 cells), cells susceptible to Coxsackie A (such as MRC-5, RD, HeLa, HEp-2, pMK, MDCK, and E-Mix cells), cells susceptible to Echovirus (such as HEL-299, HFL-Chang, and pMK cells), cells susceptible to Encephalitis (such as CV1 cells), cells susceptible to Herpesviruses (such as Human Fetal Tonsil, L-929, CHO-K1 cells), cells susceptible to HSV (such as A549, BGMK, CV1, Duck Embryo, EBTr, ELVIS-HSV, H&V-Mix, HEL, HEL-299, HeLa, HEp-2, Hs27 (HFF), LLC-MK2, MDCK, MRC-5, MRHF, Mv1Lu, NCI-H292, pAGMK, pCMK, pRK, RD, RK, RK1, R-Mix, Vero, WI 38 cells), cells susceptible to Influenza (such as A549, Chicken embryo, HEp-2, LLC-MK2, MDCK, MRC-5, pAGMK, pCMK, pRhMK, R-Mix, WI 38, NCI-H292, and Mv1Lu cells), cells susceptible to Measles (such as A549, Chicken embryo, CV1, HEp-2, LLC-MK2, NCI-H292, pMK, and Vero cells), cells susceptible to Mumps (such as A549, BGMK, CV1, HEp-2, Hs27 (HFF), LLC-MK2, MRC-5, pCMK, pMK, pRK, RK1, Vero, and WI 38 cells), cells susceptible to Myxovirus (such as LLC-MK2 cells), cells susceptible to Newcastle disease (such as Chicken embryo cells), cells susceptible to Panleukopenia (such as CHO-K1 cells), cells susceptible to Parainfluenza (such as A549, BGMK, HEp-2, Hs27 (HFF), L-929, LLC-MK2, MDCK, MRC-5, MRHF, pAGMK, pCMK, pRhMK, R-Mix, Vero, WI 38, BT, EBTr cells), cells susceptible to canine Parvovirus (such as CHO-K1 cells), cells susceptible to feline Picornavirus (such as CHO-K1 cells), most fibroblast and heteroploid cell lines, MRC-5, pCMK, WI 38, HeLa, HeLa S-3, BS-C-2, CV1, Vero, LLC-MK2 cells), cells susceptible to Poxvirus (such as LLC-MK2 cells), cells susceptible to Rabies (such as CHO-K1 and L-929 cells), cells susceptible to Reovirus (such as MDCK, EBTr, CHO-K1, L-929, NCI-H292 cells), cells susceptible to Rhinovirus (such as HEL, HEL-299, HFL-Chang, Hs27 (HFF), LLC-MK2, MRC-5, WI 38, NCI-H292 cells), cells susceptible to Rotavirus (such as A549, CV1, Vero cells), cells susceptible to RSV (such as A549, BGMK, HeLa, HEp-2, Hs27 (HFF), Human Fetal Tonsil, MDCK, MRC-5, MRHF, NCI-H292, pRhMK, R-Mix, Vero, WI 38 cells), cells susceptible to Rubella (such as BHK-21, BS-C-1, HEp-2, LLC-MK2, pMK, RK13, SIRC, Vero cells), cells susceptible to SV40 (such as BS-C-3, CV1 cells), cells susceptible

to Vaccinia (such as Chicken embryo, EBTr, L-929, NCI-H292 cells), cells susceptible to Vesicular stomatitis (such as BS-C-4, Duck Embryo, HEL-299, HeLa S-5, EBTr cells), and cells susceptible to VZV (such as A549, CV1, H&V-Mix, HEL, HEL-299, HFL-Chang, HNK, Hs27 (HFF), MRC-5, MRHF, SF, Vero, WI 38, M7, pGuinea Pig Embryo cells).

Also without intending to limit the source or type of virus, the "plus-strand RNA virus" is exemplified by togavirus, flavivirus, coronavirus, and picornavirus (including Adenovirus, Enterovirus, Immunodeficiency virus, Poliovirus, and Retrovirus).

More particularly, Togaviruses are exemplified by eastern equine encephalitis virus, western equine encephalitis virus, rubella virus. A variety of infectious agents comprise the alphaviruses (a subgroup of togaviridae), including Chikungunya, Mayaro, Igbo Ora, Ross River virus, Venezuelan equine encephalitis, Eastern equine encephalitis, and Western equine encephalitis. While the encephalitides have been discussed previously (Small Group 3, Neurotropic Viruses, November 13-15, 2001), this group of "emerging viruses" causes a range of diseases (from acute arthropathy to systemic febrile illness) in various parts of the world including the United States. A wide range of animals are hosts for these viruses, including birds, rodents, primates, wallabies, equines, and bats. However, all alphaviruses pathogenic for humans replicate in and are transmitted by mosquitoes.

Flaviviruses are exemplified by Dengue fever virus, Yellow fever virus, St. Louis encephalitis virus, Japanese B encephalitis virus, West Nile virus, and Hepatitis C virus.

The term "coronavirus" refers to a virus whose genome is plus-stranded RNA of about 27 kb to about 33 kb in length depending on the particular virus. The virion RNA has a cap at the 5' end and a poly A tail at the 3' end. The length of the RNA makes coronaviruses the largest of the RNA virus genomes. In one embodiment, coronavirus RNAs encode: (1) an RNA-dependent RNA polymerase; (2) N-protein; (3) three envelope glycoproteins; plus (4) three non-structural proteins. These coronaviruses infect a variety of mammals & birds. They cause respiratory infections (common), enteric infections (mostly in infants >12 mo.), and possibly neurological syndromes. Coronaviruses are transmitted by aerosols of respiratory secretions. Coronaviruses are exemplified by, but not limited to, human enteric CoV (ATCC accession # VR-1475), human CoV 229E (ATCC accession # VR-740), human CoV OC43 (ATCC accession # VR-920), and SARS-coronavirus (Center for Disease Control).

Picornavirus comprises several genuses such as Enterovirus (exemplified by human enterovirus A, B, C, and D, porcine enterovirus A and B, Poliovirus, Coxsackie A and B

virus, and Echo virus), Rhinovirus (exemplified by Human rhinovirus), Hepatovirus (exemplified by Hepatitis A virus), Cardiovirus (exemplified by Encephalomyocarditis virus), Aphthovirus (exemplified by Foot-and-mouth disease virus), Parechovirus (exemplified by Human parechovirus), Erbovirus (exemplified by Equine rhinitis B virus), Kobuvirus (exemplified by Aichi virus), Hepatovirus, and Teschovirus (exemplified by Porcine teschovirus).

Cells susceptible to plus-sense RNA viruses are exemplified by, but not limited to, cells susceptible to Adenovirus (such as 293, A549, HEL, HEL-299, HEp-2, HFL-Chang, HNK, Hs27, KB, LC-MK2, MDCK, MRC-5, MRHF, NCI-H292, pRK, RK1, R-Mix, Vero, WI 38, HeLa, and HeLa S-4 cells), cells susceptible to Bovine adenovirus (such as BT cells), cells susceptible to Bovine enterovirus (such as BT cells), cells susceptible to Enterovirus (such as A549, BGMK, Caco-2, HEL, HEp-2, HNK, Hs27 (HFF), LLC-MK2, MRC-5, MRHF, NCI-H292, pAGMK, pCMK, pRhMK, RD, Vero, and WI 38 cells), cells susceptible to feline Calicivirus (such as CHO-K1 cells), cells susceptible to Poliovirus (such as A549, BGMK, FL Amnion, HEL-299, HEp-2, HFL-Chang, Hs27 (HFF), and cells susceptible to bovine Infectious rhinotracheitis virus (such as BT and EBTr cells).

The invention further provides a method for detecting a virus that is not a plus-strand RNA virus in a sample, comprising: a) providing: i) a sample; ii) cells susceptible to the virus that is not a plus-strand RNA virus; and iii) one or more protease inhibitor; b) contacting the cells and the sample in the presence of the protease inhibitor to produce contacted cells, wherein replication of the plus-strand RNA virus in the contacted cells is not reduced relative to replication of the virus that is not a plus-strand RNA virus in cells not contacted with the protease inhibitor, and wherein replication of a plus-strand RNA virus in the cells contacted with the protease inhibitor is reduced relative to replication of the plus-strand RNA virus in cells not contacted with the protease inhibitor.

In one embodiment, the invention provides compositions and methods for reducing infection with SARS-coronavirus, without substantially reducing infection with other respiratory viruses. Thus, the invention provides a composition comprising (i) cells susceptible to a virus chosen from influenza virus, parainfluenza virus, adenovirus, metapneumovirus, and respiratory syncytial virus, and (ii) protease inhibitor. The invention also provides a method for detecting a virus chosen from influenza virus, parainfluenza virus, adenovirus, and respiratory syncytial virus in a sample, comprising: a) providing: i) a sample; ii) cells susceptible to the virus; and iii) one or more protease inhibitor; b)

contacting the cells and the sample in the presence of the protease inhibitor to produce contacted cells, wherein infection of the contacted cells by the virus is not reduced relative to cells not contacted with the protease inhibitor, and wherein infection of the contacted cells by severe acute respiratory syndrome coronavirus (SARS-coronavirus) is reduced relative to cells not contacted with the protease inhibitor.

These methods are premised, at least in part, on the inventors' discovery that protease inhibitors do not substantially reduce infection of cells by the exemplary respiratory viruses influenza, parainfluenza, RSV, and adenovirus (Example 8). This is in contrast to the inhibition in replication of SARS-coronavirus by the cysteine proteinase inhibitor (2S,3S)transepoxysuccinyl-*L*-leucylamido-3-methylbutane ethyl ester (Yount et al. PNAS 100:12995-13000 (2003).

The invention's methods are useful, where it is desirable to reduce infectivity by SARS-coronavirus of cells that are routinely used in diagnostic assays of respiratory viruses such as influenza, parainfluenza, RSV, and adenovirus.

As used herein the term "influenza virus" refers to members of the orthomyxoviridae family of enveloped viruses with a segmented antisense RNA genome (Knipe and Howley (eds.) Fields Virology, 4th edition, Lippincott Williams and Wilkins, Philadelphia, PA [2001]). Two types of influenza virus (A and B) are human pathogens causing respiratory pathology. While not intending to limit the type of influenza virus, in one embodiment, the influenza virus is chosen from influenza A, influenza B, and influenza C. Also while not intending to limit the cell type, the cells susceptible to influenza virus comprise cells chosen from A549 (Influenza), Chicken embryo (Influenza), HEp-2 (Influenza), LLC-MK2 (Influenza), MDCK (Influenza), MRC-5 (Influenza), pAGMK (Influenza), pCMK (Influenza), pRhMK (Influenza), R-Mix (Influenza), WI 38 (Influenza A), NCI-H292 (Influenza A), Mv1Lu (Influenza A,B), and Mv1Lu-hF (Influenza A,B). These cells are available from Diagnostic Hybrids, Inc., Athens, OH.

As used herein, the term "parainfluenza virus" refers to certain members of the paramyxoviridae genus of enveloped viruses with a single-stranded antisense RNA genome (Knipe and Howley (eds.) Fields Virology, 4th edition, Lippincott Williams and Wilkins, Philadelphia, PA [2001]). Four types of parainfluenza virus (1 to 4) are human respiratory pathogens. While not intending to limit the type of parainfluenza virus, in one embodiment, the parainfluenza virus is chosen from parainfluenza 1, parainfluenza 2, and parainfluenza 3. In another embodiment, the cells susceptible to parainfluenza virus comprise cells

chosen from A549 (Parainfluenza), BGMK (Parainfluenza), HEp-2 (Parainfluenza), Hs27 (HFF) (Parainfluenza), L-929 (Parainfluenza), LLC-MK2 (Parainfluenza), MDCK (Parainfluenza), MRC-5 (Parainfluenza), MRHF (Parainfluenza), pAGMK (Parainfluenza), pCMK (Parainfluenza), pRhMK (Parainfluenza), R-Mix (Parainfluenza), Vero (Parainfluenza), WI 38 (Parainfluenza), BT (Parainfluenza 3), and EBTr (Parainfluenza 3). These cells are available from Diagnostic Hybrids, Inc., Athens, OH.

As used herein, the term "adenovirus" refers to a double-stranded DNA adenovirus of animal origin, such as avian, bovine, ovine, murine, porcine, canine, simian, and human origin. Avian adenoviruses are exemplified by serotypes 1 to 10, which are available from the ATCC, such as, for example, the Phelps (ATCC VR-432), Fontes (ATCC VR-280), P7-A (ATCC VR-827), IBH-2A (ATCC VR-828), J2-A (ATCC VR-829), T8-A (ATCC VR-830), and K-11 (ATCC VR-921) strains, or else the strains designated as ATCC VR-831 to 835. Bovine adenoviruses are illustrated by those available from the ATCC (types 1 to 8) under reference numbers ATCC VR-313, 314, 639-642, 768 and 769. Ovine adenoviruses include the type 5 (ATCC VR-1343) or type 6 (ATCC VR-1340). Murine adenoviruses are exemplified by FL (ATCC VR-550) and E20308 (ATCC VR-528). Porcine adenovirus (5359) may also be used. Adenoviruses of canine origin include all the strains of the CAVI and CAV2 adenoviruses [for example, Manhattan strain or A26/61 (ATCC VR-800) strain]. Simian adenoviruses are also contemplated, and they include the adenoviruses with the ATCC reference numbers VR-591-594, 941-943, and 195-203. Human adenoviruses, of which there greater than fifty (50) serotypes are known in the art, are also contemplated, including the Ad2, Ad3, Ad4, Ad5, Ad7, Ad9, Ad12, Ad17, and Ad40 adenoviruses.

Without limiting the type of cell, the cells susceptible to adenovirus comprise cells chosen from 293 (Adenovirus), A549 (Adenovirus), HEL (Adenovirus), HEL-299 (Adenovirus), HEp-2 (Adenovirus), HFL-Chang (Adenovirus), HNK (Adenovirus), Hs27 (HFF) (Adenovirus), KB (Adenovirus), LLC-MK2 (Adenovirus), MDCK (Adenovirus), MRC-5 (Adenovirus), MRHF (Adenovirus), NCI-H292 (Adenovirus), pRK (Adenovirus), RK1 (Adenovirus), R-Mix (Adenovirus), Vero (Adenovirus), WI 38 (Adenovirus), HeLa (Adenovirus 3), and HeLa S-4 (Adenovirus 5). These cells are available from Diagnostic Hybrids, Inc., Athens, OH.

In another embodiment, the cells susceptible to respiratory syncytial virus comprise cells chosen from A549, BGMK, HeLa, HEp-2, Hs27 (HFF), Human Fetal Tonsil, MDCK,

MRC-5, MRHF, NCI-H292, pRhMK, R-Mix, Vero, and WI 38. These cells are available from Diagnostic Hybrids, Inc., Athens, OH.

In one embodiment, the goal of reducing infection of cells by SARS-coronavirus while not substantially reducing susceptibility of the cells to influenza, parainfluenza, RSV, and/or adenovirus viruses may be attained by contacting the cells and/or sample that is being tested with a "protease inhibitor," *i.e.*, an agent that reduces the activity of an enzyme that degrades proteins by hydrolysing peptide bonds between amino acid residues. Exemplary protease inhibitors include, but are not limited to, those obtained from Sigma, and listed in catalog 2000-20001, page 845, including AMASTATIN (page 1046), (2S, 3R)-3-Amino-2-hydroxy-4-(4-nitrophenyl)-butanoyl-L-leucine (NITROBESTATIN) (page 1046), 4-Amidinophenylmethanesulfonyl Fluoride (AMPSF) (page 84), Antipain (page 1046), α_1-Antitrypsin (page 125), Aprotinin (page 128), BESTATIN (page 160), CHYMOSTATIN (page 1046), CYSTATIN (page 299), 3,4-Dichlorolsocoumarin (page 336), EBELACTONE A (page 382), EBELACTONE B (page 382), ELASTATINAL (page 1047), trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) (page 393), ethylene diamine tetra-acetic acid (EDTA) (page 1768), EGTA (page 408), Leupeptin (page 1047), α_2-Macroglobulin (page 626), Nle-Sta-Ala-Sta (page 1047), Pepstatin A (page 1048), phenylmethylsulfonylfluoride (PMSF) (page 772), N-(α-Rhamnopyranosyloxy)hydroxy-phosphinyl-Leu-Trp (PHOSPHORAMIDON) (page 1048), TLCK (page 964), TPCK (page 964), Trypsin Inhibitor (Soybean) (page 1735), Trypsin Inhibitor (Egg) (page 992), Actinonin (page 128), and Glycyrrhizio Acid (page 489).

In one embodiment the protease inhibitor is a drug that has been approved by the FDA. These are exemplified by protease inhibitors approved for reducing infection with human immunodeficiency virus (HIV), such as, without limitation AGENERASE (AMPRENAVIR), CRIXIVAN (INDINAVIR), FORTOVASE (SAQUINAVIR), INVIRASE (SAQUINAVIR), KALETRA (LOPINAVIR), LEXIVA (FOSAMPRENAVIR) which is formerly know as GW-433908 and VX-175 and is an improved formulation of AGENERASE (AMPRENAVIR), NORVIR (RITONAVIR), REYATAZ (ATAZANAVIR; BMS-232632), and VIRACEPT (NELFINAVIR).

In one embodiment, the cells used in the invention's methods may comprise a transgenic cell, such as Mv1Lu-hF. In a further embodiment, the contacting further comprises contacting the cells with antibody specific for one or more SARS-coronavirus antigen.

The cells may be in single cell type culture or in mixed cell type culture with a second cell type. The second cell type may comprise a wild type cell and/or a transgenic cell. In one embodiment, the mixed cell types comprise mink lung cells such as Mv1Lu cells, and the second cell type comprises A549 cells (R-mix). In a further embodiment, the cells are frozen *in situ*, regardless of whether they are in single cell culture or in mixed cell culture.

Where mink lung cells such as Mv1Lu cells are used, the inoculated cells may be incubated with the sample for up to 24 hours to reduce the chance of detecting SARS, while maximizing detection of influenza virus and/or parainfluenza. Data herein (Figure 4B) shows that SARS was not produced by Mv1Lu cells within 24 hours p.i.

The samples that may be used in the invention's methods may be isolated from a mammal such as human, non-human primate, canine, feline, porcine, murine, bovine, avian, hamster, or mink.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLE 1

Materials and Methods

The following is a brief description of the exemplary materials and methods used in the subsequent Examples.

A. Virus

A seed stock of SARS-CoV Urbani that was passaged twice in Vero E6 cells provided by the Centers for Disease Control and Prevention, Atlanta, GA. This virus was amplified by two passages in Vero E6 cells to establish a high titer stock (passage 4) that was utilized for all experiments. SARS-CoV was titered in Vero E6 cells by $TCID_{50}$. Briefly, cells were plated in 96-well plates (Falcon, Becton Dickson) at a density of 4×10^5 cells/well in 150 µl of medium. Virus was serially diluted by half logs from $10^0 - 10^{-7}$, in culture medium containing 2% antibiotic-antimycotic (Invitrogen Corporation, Carlsbad, CA). 100 µl of each dilution was added per well and cells were incubated 3-4 days at 37°C.

B. Cell lines

The following Table lists exemplary cell lines that were used and/or equivalent cells that may be used in the invention's methods, and that are publicly available (*e.g.*, from the American Type Culture Collection (ATCC), Rockville, Maryland, and Diagnostic Hybrids, Inc. (DHI), Athens, Ohio; Cell Bank, Ministry of Health and Welfare, Japan): R-Mix (R-Mix FreshCells™, Diagnostic Hybrids, Inc., Ohio) is a mixed monolayer of mink lung cells (strain Mv1Lu) and human Adenocarcinoma cells (strain A549). The hAPN expression construct used to create BHK21/hAPN and CMT-93/hAPN was previously described (Wentworth, et al., 2001). Further description of Huh-7 cells is in Nakabayashi et al., Cancer Res., 42: 3858-3863, 1982; Nakabayashi et al., Gann, 75: 151-158, 1984; and Nakabayashi et al., Cancer Res., 45:6379-6383, 1985.

Table 3. Exemplary Cells Useful in the Methods and Composition of the Present Invention

Cells	Source
Vero E6	ATCC # CRL-1586; DHI # 67-0102
MRC-5	ATCC # CCL-171; DHI # 51-0102
BHK-21	ATCC # CCL-10; DHI # 89-0102
MDCK	ATCC # CCL-34; DHI # 83-0102
HRT-18 (HCT-18)	ATCC # CCL-244
Mv1Lu	ATCC # CCL-64; DHI # 58-0102
CMT-93	ATCC # CCL-223
AK-D	ATCC # CCL-150
A549	ATCC # CCL-185; DHI # 56-0102
HEL	DHI # 88-0102
pRHMK	DHI # 49-T025; DHI # 49-0102

pCMK	DHI # 47-T025; DHI # 47-0102
L2	ATCC # CCL-149
R-Mix	DHI # 96-T025
HEK-293T	ATCC # CRL-1573; CRL- 11264, CRL-11270; Pear et al., PNAS USA, Vol 90, pp 8392-8396, 1993; DuBridge et al., Mol. Cell. Biol. Vol 7, pp 379-387, 1987; University Dr. Yoshi Kawaoka, Univ. Wisconsin, Madison.
Huh-7 (JTC-39)	CellBank #JCRB0403

Vero E6, 293T, L2, AK-D, A549, pCMK, pRhMK, Mv1Lu, CMT-93, and R-mix were maintained in Dulbecco's modified Eagle Medium (DMEM) (Invitrogen Corp.) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 2% antibiotic-antimycotic. MDCK cells were maintained in DMEM high glucose (Invitrogen Corp.) supplemented with 5% FBS and 2% antibiotic-antimycotic. HEL cells were maintained in Modified Eagle's Medium (MEM) supplemented with 10% FBS and 2% antibiotic-antimycotic. HRT-18 cells were maintained in RPMI 1640 (Invitrogen Corp.) supplemented with 10% horse serum (Hyclone), 1 mM MEM sodium pyruvate (Invitrogen Corp.) and 2% antibiotic-antimycotic. Huh-7 cells were maintained in DMEM supplemented with 20% FBS and 2% antibiotic-antimycotic. MRC-5 cells were maintained in MEM supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids (Invitrogen Corp.) and 2% antibiotic-antimycotic. BHK-21 cells were maintained in DMEM supplemented with 10% FBS and 5% tris phosphate buffer (Invitrogen Corp.).

C. PCR assay

G3PDH, genomic SARS-CoV RNA (gRNA) and subgenomic RNA (sgRNA) were detected using multiplex one-step RT-PCR. Oligonucleotide primers used to amplify the different targets were as follows: G3P-279 (sense) 5' CATCACCATCTTCCAGGAGC-3' (SEQ ID NO:72) binds at nt 279-299; G3P-1069R (antisense) 5'-CTTACTCCTTGGAGGCCATG –3' (SEQ ID NO:73) binds at nt 1069-1049; SARS-

21,263 (sense) 5'- TGCTAACTACATTTTCTGGAGG-3' (SEQ ID NO:74) binds at nt 21,263-21,284 of SARS-Urbani; SARS-21,593R (antisense) 5'-AGTATGTTGAGTGTAATTAGGAG-3' (SEQ ID NO:75) binds at nt 21,593-21,571 of SARS-Urbani; and SARS-1 (sense) 5'- ATATTAGGTTTTTACCTACCCAGG-3' (SEQ ID NO:76) binds at nt 1-24 of SARS-Urbani. Amplification was carried out using the Qiagen® OneStep RT-PCR kit (Qiagen) according to the manufacturer's protocol. Briefly, each reaction consisted of 2 μg of total RNA isolated using TRIZOL® Reagent (Invitrogen), 400 μM dNTPs, 200 nM of each G3PDH primer, 400 nM SARS-1, 400 nM SARS-21,263, 600 nM SARS-21,593R and 2 μl Qiagen enzyme mix. The cycling parameters were: 50°C for 30 min, 95°C for 15 min, 35 cycles of 94°C for 30 s, 57-58°C for 30 s, 72°C for 1 min, followed by 10 min at 72°C in an Eppendorf Mastercycler gradient (eppendorf). Amplification products were analyzed by electrophoresis through a 1.5% agarose gel and visualized by ethidium bromide staining. All primers were synthesized by the Molecular Genetics Core (David Axelrod Institute, Wadsworth Center, Albany, NY).

The present invention is not limited to the use of the above-referenced SARS-Urbani primers. In fact other suitable primers can easily be devised by reviewing published sequence information in combination with the teachings of the present invention. Briefly, preferred primers are generally from 15 to 25 nucleotides in length, with an annealing temperature of about 56°C or greater. In addition preferred primer sets do not have a propensity for heteroduplex formation or primer-dimer formation. Exemplary primer sets for amplifying sgRNA and gRNA from SARS-Tor2 (GenBank Accession No. AY274119) are provided in Table 4.

Table 4. Exemplary Primer For Amplifying SARS-Tor2 sgRNA and gRNA

Leader or 1st Sense primer	SEQ ID NO	Antisense Primer	SEQ ID NO	2nd Sense primer	SEQ ID NO
AGGTTTTTAC CTACCCAGGA	97	TCATCAGGAT AGTAAACCCC	102	ACATTTTCTG GAGGAACACA	107
TTTACCTACC CAGGAAAAGC	98	CCTGAGTTAA ATAAAGAGTG	103	TTCTGGAGGA ACACAAATCC	108
CTACCCAGGA AAAGCCAACC	99	GTTAAATAAA GAGTGTCTGA	104	GAGGAACACA AATCCTATCC	109
CAGGAAAAGC CAACCAACCT	100	ATAAAGAGTG TCTGAYCTAA	105	ACACAAATCC TATCCAGTTG	110
CAACCAACCT CGATCTCTTG	101	GAGTGTCTGA TCTAAAAATT	106	TATCCAGTTG TCTTTCCTATT	111

D. Cell infection

Cells seeded at a density of $2x10^6$ in T25 flasks (Falcon, Becton Dickson) were inoculated with virus at an MOI of 0.001 in a final volume of 1 ml and were incubated 1 h at 37°C. Virus was removed and 5 ml fresh medium added to each flask. Cells were maintained at 37°C throughout the experiment. At 1, 24 and 48 h post-inoculation (p.i.), cells were observed for CPE, supernatants were collected for subsequent titration and total RNA was extracted using TRIZOL® Reagent (Invitrogen Corp.). RNA was quantitated by spectrophotometer (Eppendorf).

EXAMPLE 2

Exemplary Multiplex RT-PCR Assay

For The Detection of SARS-CoV Replication

A RT-PCR assay for the detection of SARS-CoV replication was developed. Replication of corona- and arteri- virus RNA occurs through discontinuous synthesis, thought to occur during negative strand synthesis, generating 3' co-terminal nested subgenomic RNAs (sgRNA). The inventors identified targets within the genome for amplification. Oligonucleotide RT-PCR primers were designed that amplify genomic SARS-CoV RNA (gRNA) or the sgRNA that is specific to the leader-body junction. Because genomic RNA is present in input virus, the inventors probed for sgRNA, which is

indicative of virus entry and/or replication initiation. Genomic RNA was detected by amplifying a region between the 1b coding region of the polymerase gene and the sequence encoding the Spike (S) glycoprotein. Subgenomic RNA was detected using a primer specific to the leader sequence in conjunction with the reverse primer in S that was used for the gRNA detection. G3PDH primers, designed to amplify G3PDH from multiple species, served as a positive control for RNA integrity and cDNA production.

To evaluate the RT-PCR assay, Vero E6 cells were inoculated with serial dilutions of SARS-CoV ranging from a MOI of 10^0 to 10^{-8} $TCID_{50}$/cell. Total RNA was extracted at 1 and 24 h post-inoculation (p.i.). At 1 h p.i., gRNA was detected in cells inoculated with virus at an MOI of 10^0 to 10^{-2}, as indicated by a band at 300bp (Fig. 1). Subgenomic RNA was not detected (180 bp). However, at 24 h p.i., both gRNA and sgRNA, 300 bp and 180 bp respectively, were detected in cells inoculated with an MOI of 10^0 to 10^{-5}. The sgRNA amplicon was confirmed to correspond to the S leader-body junction sgRNA by sequence analysis (Thiel, et al., 2003, J.Gen.Virol. 84:2305-2315). Genomic RNA was visible at 24 h p.i. in cells inoculated with an MOI of 10^{-7}, however this was not seen in repeated experiments. The decrease in amplified G3PDH (~800bp) as seen in lanes 1-6 at 24 h p.i. was consistent between repeated experiments. The decrease in G3PDH may be a result of the RT-PCR conditions, which were optimized to favor amplification of SARS-CoV gRNA and sgRNA. Individual amplicon were amplified by PCR of cDNAs from the same samples and G3PDH was consistently detected. Additionally, the decrease in G3PDH may be due to cell death, which is seen in Vero E6 cells. G3PDH was included as a control for template concentration and RNA integrity, and was always detected in the absence of viral RNA.

This data demonstrates that the exemplary multiplex RT-PCR assays are sufficiently sensitive for detection of SARS-CoV infection.

EXAMPLE 3

Primary Monkey Kidney Cells pRhMK and pCMK Are Susceptible and Permissive To SARS-CoV

To test the specificity of the RT-PCR assay and to identify cells susceptible to SARS-CoV, kidney cells derived from two species of monkey were inoculated with SARS-CoV at an MOI of approximately 0.001. Vero E6 cells were included in all experiments as a positive control. Entry and early replication of SARS-CoV was detected in primary Rhesus monkey kidney cells (pRhMK) and primary Cynomolgus monkey kidney cells

(pCMK) at 24 and 48 h p.i. (Fig. 2A). SARS-CoV genomic RNA was detected at 1 h p.i. and increased by 24 and 48 h p.i.. In both cell types, sgRNA, absent from input virus (1 h) was detected at 24 and 48 h p.i.. Once again, G3PDH amplification decreased as the amplification of viral RNA increased. Subgenomic RNA was not detected in inoculated baby hamster kidney cells (BHK-21), included as a negative control. In these cells, gRNA was detected only in the viral inoculum (1 h). Both pRhMK and pCMK support productive SARS-CoV infection as demonstrated by virus titration (Fig. 2B). Inoculated cells demonstrated an increase in viral titer by 48 h p.i. over input virus (2.5 log and 1.5 log increases respectively). At 48 h p.i. supernatants from pRhMK contained virus titers of 5.6×10^5 $TCID_{50}$ and supernatants from pCMK contained virus at titers of 7.8×10^4 $TCID_{50}$. The rise in titer was different between the two cell types; pCMK demonstrated a slower rise in titer from 1 to 24 h p.i. than both pRhMK and Vero E6. Supernatants collected from Vero E6 cells at 48 h p.i. contained viral titers of 3.9×10^7 $TCID_{50}$. Consistent with reports by Ksiazek et al., cytopathic effect (CPE) was observed in Vero E6 cells as early as 24 h p.i. (Ksiazek, et al., 2003, N.Engl.J.Med. 348:1953-1966). Surprisingly, however, significant CPE was not observed in pRhMK or pCMK 5 days p.i.

This data suggest that CPE may not be an accurate indicator of SARS-CoV replication. Furthermore, this is the first report of the susceptibility and permissivity of primary monkey kidney cells pRhMK and pCMK to SARS-CoV.

EXAMPLE 4

Cells Expressing Known Coronavirus Receptors Are Not Susceptible to SARS-CoV

Cell lines known to be susceptible to other coronaviruses were assayed for their susceptibility to SARS-CoV. Human, feline, canine and murine cells expressing known coronavirus receptors were inoculated with SARS-CoV and assayed for viral replication. Cells expressing the receptor for serogroup 1 coronaviruses (APN) tested included human lung fibroblast-derived cells (MRC-5), canine kidney-derived cells (MDCK), and feline lung epithelia (AK-D). These cells are susceptible to human coronavirus 229E (HCoV-229E), canine coronavirus (CCoV), and feline coronavirus (FcoV), respectively. Cells permissive to group 2 coronaviruses were also analyzed, including mouse fibroblast derived cells (L2), that expresses CEACAM 1a, the receptor utilized by MHV-A59 and MHV-JHM and a human rectal tumor cell line (HRT-18), known to be susceptible to HCoV-OC43.

SARS-CoV gRNA was amplified in all four cell lines at 1, 24 and 48 h p.i. (Fig. 3) ; however, sgRNA was not detectable at any time points post inoculation. A non-specific band (~220 bp) was amplified in MRC-5, MDCK and AK-D cells, in all samples including mock. Subgenomic RNA was detected in Vero E6 cells included as a positive control. This data suggest that SARS-CoV utilizes a different receptor than both group 1 and group 2 coronaviruses.

EXAMPLE 5

Mv1Lu Cells Are Susceptible and Permissive to SARS-CoV

Virology laboratories routinely inoculate cells with clinical specimen to identify potential respiratory pathogens. Because little is known about the cell types susceptible to SARS-CoV, cells utilized by clinical laboratories were assayed. R-Mix, a mixed monolayer of mink lung-derived cells (Mv1Lu) and human lung-derived cells (A549) are used to detect a range of respiratory pathogens. Influenza A and B, adenovirus, RSV and parainfluenza can be detected in Mv1Lu cells while influenza and adenovirus can be detected in A549 cells. Human embryonic lung cells (HEL) are often used to detect rhinovirus and RSV. R-Mix, Mv1Lu, A549 and HEL were inoculated with SARS-CoV at an MOI of 0.001. SARS-CoV genomic RNA was detected in all four cell lines at 1, 24 and 48 h p.i. (Fig. 4A); however, while the gRNA increased from 1 to 48 h p.i. in R-Mix and Mv1Lu cells, it decreased in A549 and HEL cells (Fig. 4). Subgenomic RNA was amplified in R-Mix and Mv1Lu cells at 24 and 48 h p.i. but was not detectable in A549 and HEL cells at any time points post inoculation. These results suggest that Mv1Lu cells support productive SARS-CoV infection. A non-specific band (~220 bp) was amplified in all four cell lines but was present in all samples including the mock infection. Supernatants from R-Mix and Mv1Lu cells were titered on Vero E6 cells (Fig. 4B). Viral titers decreased approximately 0.5 log from 1 h to 24 h p.i. and then increased 1.5 logs by 48 h p.i.. Viral titers from Vero E6 cells increased sharply by 4 logs from 1 to 24 h p.i., and then leveled off. Data herein shows that while SARS-CoV can productively infect Mv1Lu cells, viral replication occurs at much lower levels than that observed in Vero E6 cells.

This is the first report of the susceptibility and permissivity of Mv1Lu cells to SARS-CoV.

EXAMPLE 6

Human Cell Lines HEK-293T and Huh-7 Are Susceptible and Permissive to SARS-CoV

Although humans have been infected by SARS-CoV, human-derived cells susceptible to SARS-CoV infection have not been reported. Human embryonic kidney-derived cells (HEK-293T) and human liver-derived cells (Huh-7) were inoculated with SARS-CoV at an MOI of 0.001. SARS-CoV gRNA was detected at 1, 24 and 48 h p.i. in both cell lines, and increased from 1 to 24 h p.i. (Fig. 5A). Subgenomic RNA was amplified at 24 and 48 h p.i. in both HEK-293T and Huh-7 cells, indicating that they were permissive to SARS-CoV infection. MDCK cell, included as a negative control, were negative for sgRNA at all time points. Supernatants collected at all time points were titered on Vero E6 cells (Fig. 5B). A 2-log increase in viral titer ($TCID_{50}$) was seen at 48 h p.i. in Huh-7 cells while an increase of less than 1 log was seen in 293T cells, compared to a 4 log increase in Vero E6 cells. CPE was apparent by 24 h p.i. in Vero E6 cells inoculated at the same time however, no CPE was observed in Huh-7 or HEK-293T cells out to 48 h p.i.. Surprisingly, these results again suggest that CPE is not an accurate indicator of viral replication in all cell lines.

This is the first report of human cell lines that are susceptible and permissive to SARS-CoV.

EXAMPLE 7

Transgenic Cells Expressing Aminopeptidase N Are Not Permissive to SARS-CoV

As demonstrated above, MRC-5 cells did not support SARS-CoV RNA replication suggesting that APN is not sufficient to render cells permissive to SARS-CoV. However, the human cell lines HEK-293T and Huh-7, shown to be permissive to SARS-CoV replication, express hAPN, the host cell receptor utilized by HCoV-229E. To further test the role of APN in SARS-CoV entry, cells expressing relatively high levels of hAPN on their surface were tested for susceptibility to infection with SARS-CoV. The murine epithelia-derived cell line (CMT-93) and the baby hamster kidney cell line (BHK-21) were transfected with constructs expressing hAPN to yield CMT-93/hAPN and BHK-21/hAPN (Wentworth et al. 2001. J.Virol. 75:9741-9752). These cells, normally non permissive to HcoV-229E infection and replication, were rendered permissive to infection by expression

of hAPN. SARS-CoV genomic RNA was detected in CMT-93, CMT-93/hAPN, BHK-21 and BHK-21/hAPN cells inoculated with SARS-CoV, at 1 to 24 h p.i. (Fig. 6A). The presence of genomic RNA at time points post inoculation varied between experiments. SARS-CoV subgenomic RNA was not detected at any time points demonstrating that all four cell lines were non permissive for SARS-CoV replication. Human APN was expressed at high levels on both CMT-93/hAPN and BHK-21/hAPN cells as demonstrated by FACS (Fig. 6B). Additionally, Huh-7 cells, included as a positive control for SARS-CoV replication, also express high levels of hAPN as demonstrated by FACS analysis.

EXAMPLE 8

Protease Inhibitors Do Not Reduce Infection of Cells by the Respiratory Viruses Influenza, Parainfluenza, and Adenovirus

This Example describes the effect of exemplary protease inhibitors on the detection of influenza A & B, RSV, adenovirus, and parainfluenza 1, 2, and 3 in R-Mix cells.

A. Materials and Methods

Viruses used were influenza A, influenza B, RSV, adenovirus, parainfluenza 1, 2, and 3, all contained in the respiratory virus proficiency panel (Diagnostic Hybrids, Inc., Athens, OH). Protease inhibitors tested were Actinonin, a leucine aminopeptidase inhibitor (Sigma), Glycyrrhizin, a biologically active derivative of licorice root (Sigma), and E-64, a cysteine protease inhibitor (Sigma). Cells were R-Mix (Diagnostic Hybrids, Inc., Athens, OH) in 48 well plates. Medium was RM03T (Diagnostic Hybrids, Inc., Athens, OH). Viral detection was by monoclonal antibody specific for the viruses tested, using a "D3" antibody kit (Diagnostic Hybrids, Inc., Athens, OH).

B. Procedure

All inhibitors were dissolved it RM03T to give final concentrations of: Actinonin, 40, 20, and 10 mcg/ml, Glycycrrhizin, 6.08, 1.216, and 0.152 mcg/ml, and E-64, 10, 5, 0.5 mcg/ml. Viral stocks were diluted in RM03T. R-Mix 48 well plates containing the appropriate concentration of inhibitor and additional no-inhibitor control wells were inoculated with the seven individual viruses separately. The inhibitor wells were in duplicate and the control wells were six replicates for each virus. Following inoculation, all plates were centrifuged at 700g for one hour at room temperature, then incubated in a

humidified, CO_2 incubator at 37^0 C for 24 hours. The cell monolayers were then fixed and stained according to the D3 detection kit instructions. Infected foci were counted using fluorescent microscopy. The data is shown in Table 5.

Table 5. Number of Infected Foci In R-Mix Cell Cultures Contacted With a Respiratory Virus in the Presence of a Protease Inhibitor[1]

Virus		Control*	Actinonin			Glycycrrhizin			E64		
		0	40**	20	10	6.08	1.216	0.152	10	5	0.5
FluA		189 ± 012	118	122	171	163	175	218	176	179	174
FluB		455 ± 022	190	260	317	273	402	426	323	328	398
Adeno		863 ± 100	147	383	781	1061	938	766	865	868	904
RSV		116 ± 012	41	64	103	53	103	130	103	115	107
Para1		245 ± 018	160	191	222	295	233	268	207	243	251
Para2		243 ± 012	151	196	220	281	257	265	205	221	223
Para3		142 ± 014	75	104	115	138	149	138	107	99	127

[1] Concentration of protease inhibitors is shown in micrograms/ml, * mean of 6 samples; **Evidence of cell toxicity; R-Mix lot 960925.

The above results demonstrate that protease inhibitors are not inhibitory to infection by any of the seven exemplary viruses that are detected by Mv1Lu cells and/or R-Mix cells. This is in contrast to the inventors' data demonstrating inhibition in replication of human coronavirus 229E by the protease inhibitor E64.

EXAMPLE 9

Detection of Human and Murine Coronaviruses By RT-PCR

Subgenomic primers were designed for the following three coronaviruses: human coronavirus 229e; human coronavirus OC43; and mouse hepatitis virus. These three coronaviruses, along with SARS, utilize the same general mechanism of replication, whereby subgenomic mRNA transcripts must be produced in order to make the viral proteins necessary for replication. Oligonucleotide primers designed against subgenomic mRNA transcripts are then used in reverse-transcriptase polymerase chain reaction (RT-PCR) to detect the presence of specific, subgenomic RNA transcripts, and thereby the

presence of replicating virus.

Briefly, a receptive host cell is inoculated with a coronavirus of interest, centrifuged and incubated at 35-37°C for a desired length of time. Cells are lysed prior to performing a standard RNA isolation protocol. Reagents are then combined for one-step, RT-PCR (buffer, water, dNTPs, subgenomic primers to 0.6μm final concentration, Taq polymerase, reverse transcriptase, Rnase inhibitor) before addition of about 1 pg to 2 μg of RNA template per reaction. Reaction mixtures are then subjected to a thermocycling program. The RT-PCR products are size-fractionated on an agarose gel (1.6%, Tris Borate EDTA) along with a standard DNA ladder (100 to 1000 bp), and then visualized via ethidium bromide staining and UV illumination.

Genomic DNA and subgenomic DNA are typically detected together in each reaction. While the presence of subgenomic RNA indicates active replication is underway, the presence of genomic RNA serves to confirm that input virus was initially present in the monolayer

All primers described below were custom synthesized by Invitrogen Corporation:

Human Coronavirus 229E

Genomic RNA is amplified using a sense primer, 229E-Pol-20,306 (5'-GGCGTAACTC CACTGTTATG A-3' set forth as SEQ ID NO:88) paired with an anti-sense primer, 229E-S-124R, complementary to bases 20655-20675 of the 229E genome (5'-AATAACCAAC ACAGCCGTTG C-3' set forth as SEQ ID NO:89). Subgenomic RNA is amplified using a sense primer, leader-1, (5'-ACTTAAGTAC CTTATCTATC TACA-3' set forth as SEQ ID NO:90) paired with the anti-sense primer 229E-S-124R.

The subgenomic amplicon sequence starts at the beginning of the 229E leader sequence (at base number 1) and concludes with the first 106 bases of the 229E Spike gene (bases 20,570-20,676). The subgenomic RNA amplification yields a RT-PCR product of about 183 bp and the genomic RNA amplification yields an approximately 316 bp band.

The present invention is not limited to the use of the above-referenced CoV 229E primers. In fact other suitable primers can easily be devised by reviewing published sequence information in combination with the teachings of the present invention. Briefly, preferred primers are generally from 15 to 25 nucleotides in length, with an annealing temperature of about 56°C or greater. In addition preferred primer sets do not have a propensity for heteroduplex formation or primer-dimer formation. Exemplary primer sets

for amplifying sgRNA and gRNA from CoV 229E can be determined as described in Example 1 for SARS-Tor2, from review of the CoV 229E sequence disclosed in GenBank Accession No. NC_002645, herein incorporated by reference.

Human Coronavirus OC43

The specific primers used to amplify genomic OC43 RNA are: OC43-ns2a (sense) bases 22,060-22,085 (5'-GTCACTGGAT GGGAATTCG -3' set forth as SEQ ID NO:94); and OC43-HE-R (antisense) bases 22,569-22,587 (5'- TGGAGTTGCC AGCTTTAG -3' set forth as SEQ ID NO:95). Subgenomic RNA from the OC43 HE protein is amplified using the sense leader primer OC43-Leader 1 (5'- GATTTGCGTG CGTGCATCCC -3' set forth as SEQ ID NO:96) and the anti-sense primer OC43-HE-R.

The subgenomic amplicon sequence starts within the OC43 leader sequence (base 10) and extends from the start of the HE gene for 230 bases (base 22,355 to 22,587). The subgenomic RNA amplification yields a RT-PCR product of about 500 bp and the genomic RNA amplification yields a band of approximately 300 bp.

The present invention is not limited to the use of the above-referenced CoV OC43 primers. In fact other suitable primers can easily be devised by reviewing published sequence information in combination with the teachings of the present invention. Briefly, preferred primers are generally from 15 to 25 nucleotides in length, with an annealing temperature of about 56°C or greater. In addition preferred primer sets do not have a propensity for heteroduplex formation or primer-dimer formation. Exemplary primer sets for amplifying sgRNA and gRNA from CoV OC43 can be determined as described in Example 1 for SARS-Tor2, from review of the CoV OC43 sequence disclosed in GenBank Accession No. NC_005147, herein incorporated by reference.

Mouse Hepatitis Virus (MHV)

The specific primers used to amplify genomic MHV RNA are: MHV-orf-3 (sense) bases 23,743-23,762 (5'-CTATGGGTAC GGTCATTGT-3' set forth as SEQ ID NO:91); and MHV-S-224R (antisense) (5'-TGGCCAGCTA CCAAGATTC-3' set forth as SEQ ID NO:92. Subgenomic RNA from the MHV spike "S" gene is amplified using: the sense primer, MHV-Leader, (5'-TACGTACCCT CTCAACTC-3' set forth as SEQ ID NO:93); and the anti-sense primer MHV-S-224R.

The subgenomic amplicon sequence starts within the MHV leader sequence, which

is approximately 72 bases long (at base number 22), and concludes with the first 225 bases of the MHV "S" gene (bases 23,929-24,154). The subgenomic RNA amplification yields a RT-PCR product of about 285 bp and the genomic RNA amplification yields a band of approximately 410 bp.

The present invention is not limited to the use of the above-referenced MHV primers. In fact other suitable primers can easily be devised by reviewing published sequence information in combination with the teachings of the present invention. Briefly, preferred primers are generally from 15 to 25 nucleotides in length, with an annealing temperature of about 56°C or greater. In addition preferred primer sets do not have a propensity for heteroduplex formation or primer-dimer formation. Exemplary primer sets for amplifying sgRNA and gRNA from MHV can be determined as described in Example 1 for SARS-Tor2, from review of the MHV strain A59 sequence disclosed in GenBank Accession No. AY700211, herein incorporated by reference.

An exemplary Reverse Transcriptase-PCR Thermocycling program for use with the primers of the present invention includes the following steps: 1) 50° C for 30 minutes; 2) 95° C for 10 minutes; 3) 95° C for 30 seconds; 4) 57° C for 30 seconds (229e) or 55°C for 30 seconds (MHV and OC43); 5) 72° C for 1 minute; 6) repeat steps 3-5 for 35 cycles; then 7) 72° C for 10 minutes.

EXAMPLE 10

Inhibition of Human Coronavirus Replication *In Vitro* With an Improved Protease Inhibitor Formulation

A modified cyclodextrin (CAPTISOL, CyDex, Overland Park, KS) is used to enhance protease inhibitor solubilization, stabilization and lyophilizability. CAPTISOL is the trade name of sulfobutyl ether beta-cyclodextrin sodium (SBECD, CAS No. 182410-00-0). Other suitable cyclodextrins for use with the methods and compositions of the present invention include but are not limited to those disclosed in U.S. Patent No. 5,134,127, and 5,376,645, herein incorporated by reference. A 20% w/v working Captisol solution is prepared in sterile, ddH_2O. The protease inhibitor E64d is dissolved in the 20% Captisol solution as a 1 mg/ml stock. The stock solution is then serially diluted (1:1) in the 20% aqueous Captisol solution until a concentration of 0.015 mg/ml E64D is obtained. To establish a dose response curve, 32 µl of each of the E64D dilutions is added to every 970 µl of culture media. This results in concentrations of E64D from 32 µg/ml to 0.5 µg/ml in

the culture media (all with a constant 3.2% Captisol concentration). Next a viral working stock is prepared by thawing a high titer master stock of Human CoV 229E, and diluting in culture media to a working titer of ~2500 virus/0.2ml. MRC-5 cell cultures are fed by aspirating plating medium from each shell vial, before adding 1ml of the appropriate E64D dilution to each MRC-5 vial. MRC-5 cell cultures are inoculated by addition of 0.2 ml of the 229E virus stock solution to the appropriate MRC-5 shell vials. After re-capping, the shell vials are centrifuged at 700xg for 1 hour, and then placed in a 32-34°C incubator. For VICP processing, cultures are incubated for 16-18 hours, while for RT-PCR processing, cultures are incubated for up to 5 days.

Viral Induced Cellular Protein (VICP) Detection

After 16-18 hours incubation, remove vials from incubator, and aspirate the media. Add 0.5ml methanol to each vial, and let set at room temperature for 10 minutes. Then aspirate methanol, and add 0.5ml of sterile PBS to each vial. After aspirating the PBS rinse, add 0.2 ml of Chemicon Pan-Entero Blend mAb and incubate for 1 hour at 35-37°C. Aspirate the primary mAb solution and rinse with 1.0 ml of PBS. Aspirate the PBS, add 0.2 ml of DHI ELVIS Solution 3, and incubate for 1 hour at 35-37°C. Aspirate Solution 3, rinse coverslip in ddH_2O and place cell side down onto a drop of DHI Mounting fluid (placed on a glass slide). Examine for fluorescent green plaques by UV microscopy.

In an exemplary 16 hour bio-assay, the protease inhibitor E64d inhibited the replication of human coronavirus 229E by 100% at concentrations of 32ug/ml to 2ug/ml. 90% inhibition was obtained with E64D concentrations of 1ug/ml and 0.5ug/ml. This result was based on the percentage of fluorescent plaques observed in the drug treated vials as compared to the 0 drug control vials.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA extractions were performed on monolayers at 1 day, 3 days and 5 days post inoculation, using a Qiagen Kit according to manufacturer's instructions. The RNA samples were stored at -20°C for subsequent use in RT-PCR as described above in Example 9.

In an exemplary RT-PCR assay, sub-genomic bands (indicative of active viral replication) were not observed after a 5 day incubation period in the presence of 32μg/ml E64D.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiment, it should be understood that the invention as claimed should not be unduly limited to such specific embodiment. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art and in fields related thereto are intended to be within the scope of the following claims.